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# THE PHILIPPINE JOURNAL OF SCIENCE

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*The cover, taken from the sixth manuscript (Figure 9) published in this issue, is a revertant colonies in Salmonella typhimurium TA 100 treated with $10^{-3}$ crude extract of Azadirachta indica A. Juss (neem) leaves.*

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# Bench Scale Production of Monolaurin from Coconut $C_{12}$ Fatty Acid

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## ABSTRACT

*A method was developed for the esterification of coconut $C_{12}$ fatty acid with glycerol to produce monolaurin in good yield and quality. The reaction parameters, i.e. molar ratio of the reactants in 1:2.5(F.A.:Gly), temperature of 180°C, 5 hours reaction time were optimized to obtain a product in 55.2% yield with 98.50% purity. Three methods of purification namely Water Refining, Low Temperature Crystallization and Molecular Distillation were studied and developed in this process. Among the three, the latter gave the highest quality monolaurin. The degree of purification of the product has been found to be a limiting factor to conform with the standard specification and requirement such as its percentage monolaurin content and its melting point for its various applications such as emulsifier, shortenings, preservatives, cosmetics, ointments and medicine. Fatty acid composition of the raw materials and the monolaurin, dilaurin and trilaurin were determined by gas chromatography. The refined monolaurin and standard monolaurin were determined and characterized by gas chromatography, high pressure liquid chromatography, infra red spectroscopy and nuclear magnetic resonance for comparison.*

## INTRODUCTION

In 1968 Babayan introduced medium chain triglycerides (MCT) which is known for its efficient and quick source of energy. Structured fats was also introduced by Babayan (1985). This structured fats with a MCT backbone with built-in essential fatty acid i.e. linoleic acid is best for patients, particularly the critically ill. These MCT and structured fats which were both prepared from the esterification of medium chain fatty acid, $C_8$ -$C_{10}$ had shown and manifested the nutritional and therapeutic effects of coconut oil. However, after several decades of research it was demonstrated that lauric acid, C:12 is one of the truly beneficial among the medium chain fatty acids.

It is the fatty acid found in human milk that is known to have unique anti-microbial properties; its presence in milk keeps infants from being infected by many viruses, protozoa, and some bacteria (Hamosh 1991, Thormar 1987). Several decades of research have demonstrated that "Lauric acid is one of the best inactivating fatty acids and its monoglyceride form is even more effective than the fatty acid alone" (Enig 1994). The digestive tract converts lauric acid into the powerful anti microbial monoglyceride called monolaurin. Monolaurin is formed in the digestive tract only when lauric acid is available in the diet (Hamosh 1991; Thormar 1987). Monolaurin inactivates envelope viruses (Thormar 1987) such as the HIV virus, cytomegalovirus, herpes virus, sna virus, as well as numerous bacteria and protozoa (Hamosh 1991) that are known to cause opportunistic infections in individuals who are immune-suppressed.

Being one of the largest region of coconut plantation and largest exporter in Asia, and coconut oil as having the largest constituent of lauric fatty acid (about 48%) which its potentials as anti-microbial, anti-viral, and anti-protozoal have not been yet explored, this study has developed and established a process for the production of a low volume but high value monolaurin from lauric acid of coconut oil. The optimum conditions for the esterification of lauric acid with glycerol were established to obtain purified Monolaurin with 55.2% yield based on the theoretical yield and 98.50% purity. The physico-chemical properties of this monolaurin were determined and evaluated in comparison with the standard specification. Purification studies were also conducted to obtain a product that conform with the specific requirements of pharmaceutical industries particularly for medicinal and therapeutic applications.

## MATERIALS AND METHODS

Lauric acid was provided by Cocochem, Philippines. Its typical analysis as starting raw material is shown in Table 1. Standard monolaurin with the commercial name of Monolauroyl-rac- Glycerol, 99% pure, is a product of Sigma and purchased from Daimar Enterprises Inc. Glycerol and other analytical reagents, i.e acetone, acetonitrile and isopropyl alcohol used in the analysis were also locally purchased from the chemical suppliers (Merck, Instruchem, Chemline,etc.).

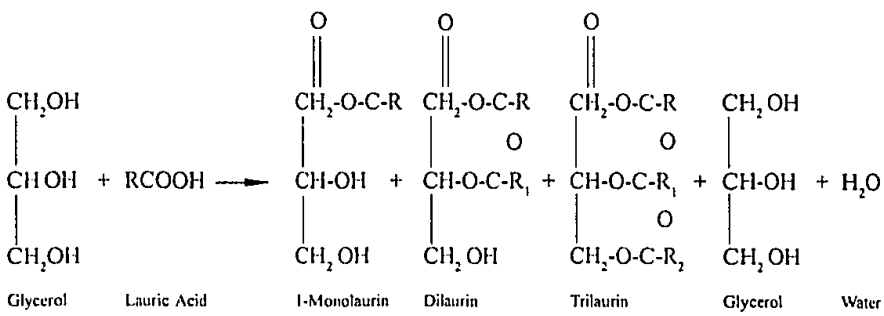
**Table 1. Typical analysis of coconut lauric acid used as starting material.**

| | |
|---|---|
| Saponification Value, mg | 279 |
| Acid Value, mg | 279 |
| Iodine Value, mg | 0.02 |
| Melting Point, °C | 43.5°C |
| Color, Lovibond | 0.6 red |
| 5¼ cell | 2.2 yellow |
| Moisture, % | 0.06 |
| GC analysis, % | |
| $C_{10}$ | 0.8 |
| $C_{12}$ | 99.0 |
| $C_{14}$ | 0.2 |

## EXPERIMENTAL PROCEDURE/METHODOLOGY

Coconut $C_{12}$ fatty acid was esterified with glycerol using the JICA donated 50-liter capacity Glass Lined Stirred Reactor. The established conditions such as the molar ratio, reaction temperature and time were used in the process. Routes in the esterification of glycerol is shown in Figure 1, while the process flowchart is shown in Figure 2.

**Figure 1. Routes in the esterification of glycerol.**




The crude product obtained from the esterification was subjected to purification processes such as the molecular distillation method, low temperature crystallyzation method and water-refining method. The comparison of percentage purities and melting points of the product is shown in Table 2.

**Table 2. Comparison of product obtained using different methods of purification.**

| Method of purification | % Purity | Melting point, °C |
|---|---|---|
| Molecular distillation method | 98.50 | 63.0°C |
| Low Temp Cystallization method | 57.00 | 43.0°C |
| Water Refining method | 55.00 | 43.0°C |

Standard monolaurin: %Purity - 99; Melting point: 63°C.

### Molecular Distillation Method

Crude monolaurin was subjected to molecular distillation method. The equipment used was Centrifugal Molecular Still, Model CEH-300B, Yamato. Three kilos of crude monolaurin was charged to the equipment. The distillation temperature for the non-reacted components was 75°C, while that for the monolaurin was 95°C.

### Low Temperature Crystallization Method

Crude Monolaurin was dissolved using acetone as solvent. One hundred grams of crude monolaurin was mixed with 400 ml acetone. The solution was stirred moderately until it attained homogeneity. The homogenous solution was immersed in a cold bath of about 15°C temperature while stirring until it crystallized. The crystallized product was filtered and air dried for 1 to 2 days.

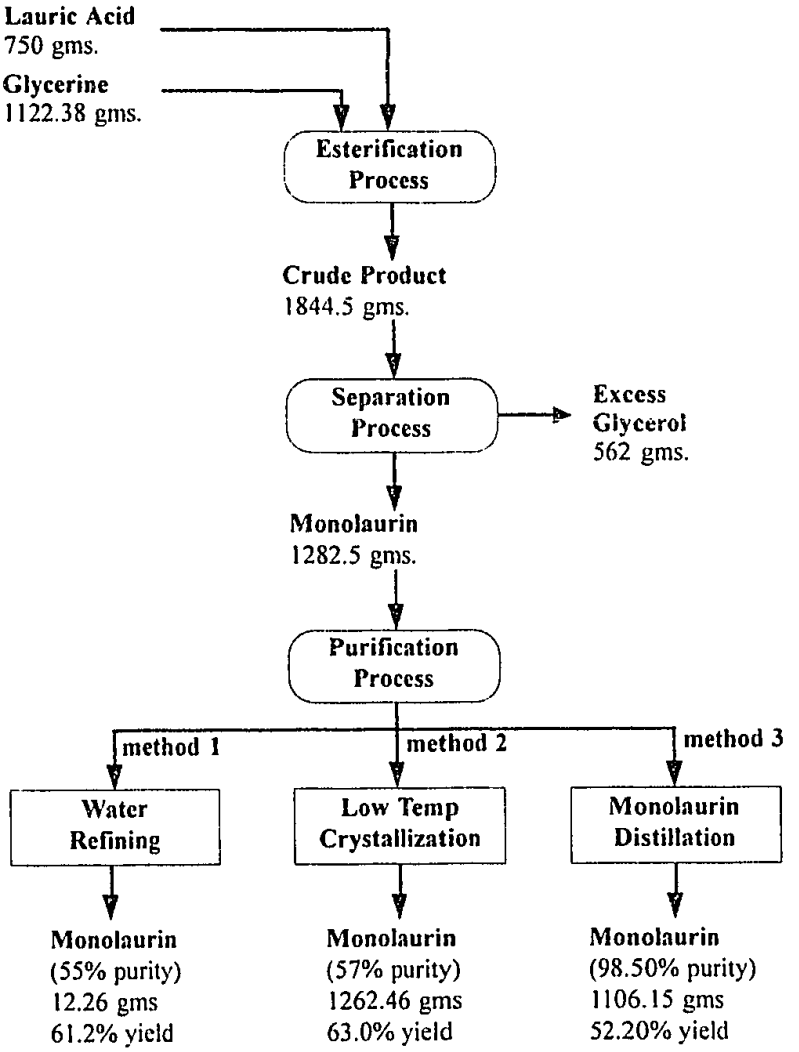
### Water-Refining Method

Crude monolaurin was washed in a separatory funnel with lukewarm brine solution (5%). The brine was added to 500 ml lukewarm water and this solution was added to the crude monolaurin, the mixture shaken and settled for a few minutes or until two layers had completely separated from the lower layer. The lower layer was discarded and an anhydrous sulfate (5%) was added to the upper layer and mixed. The mixture was then settled for about an hour. The final product was filtered and air dried for 1 to 2 days.

### Esterification of Lauric Acid with Glycerol

Complete esterification was conducted in a three-necked glass reactor with a thermometer, substripped with nitrogen gas, and a water condenser to distill out the water by product. One mole of fatty acid was reacted with an excess of the equivalent mole of the glycerol at specified reaction temperature and time. The schematic diagram of the esterification process is shown in Figure 2.

**Figure 2. Scheme of typical monolaurin processing.**

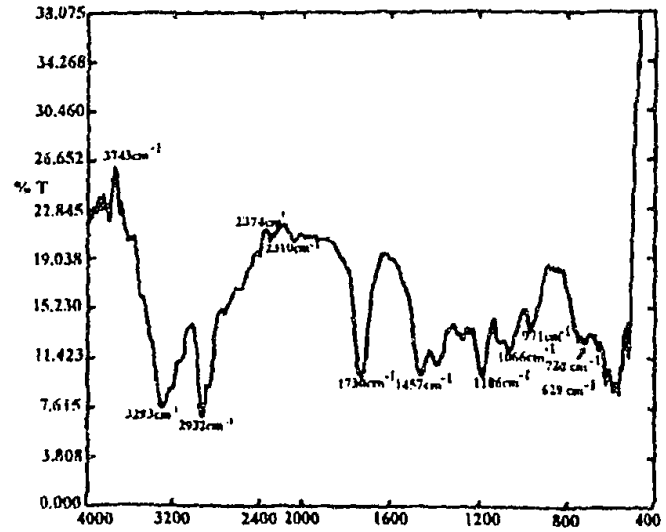



## ANALYTICAL METHODS FOR MONOLAURIN CHARACTERIZATION

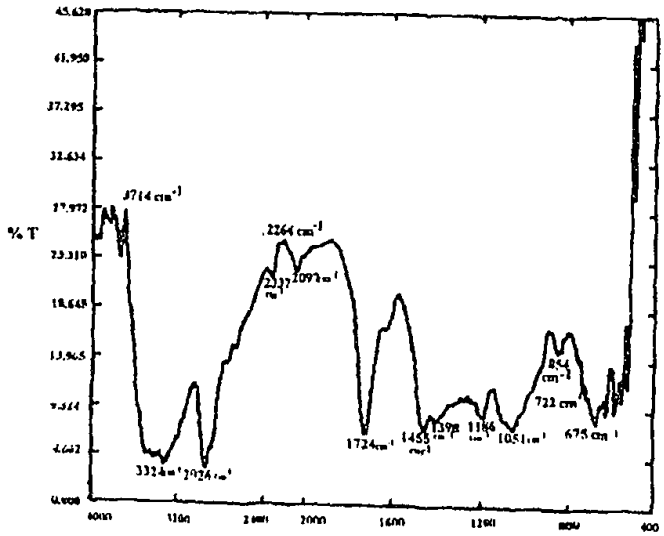
### Fourier Transform Infra Red (FTIR)

The purified monolaurin was analyzed and characterized using Perkin Elmer FTIR, model 1720x employing Diffused Reflectance Technique. FTIR spectra of both the standard and ITDI monolaurin are shown in Figure 3. The FTIR spectrum of Sigma monolaurin standard is almost identical with ITDI Monolaurin. Esterification of lauric acid into monolaurin is evident by the appearance of a sharp band at 1186 $cm^{-1}$ and a weak band at 1051 $cm^{-1}$ for the C- O - C of the monolaurin formed.

**Figure 3. Comparative spectra of standard monolaurin and ITDI monolaurin.**




Standard Monolaurin

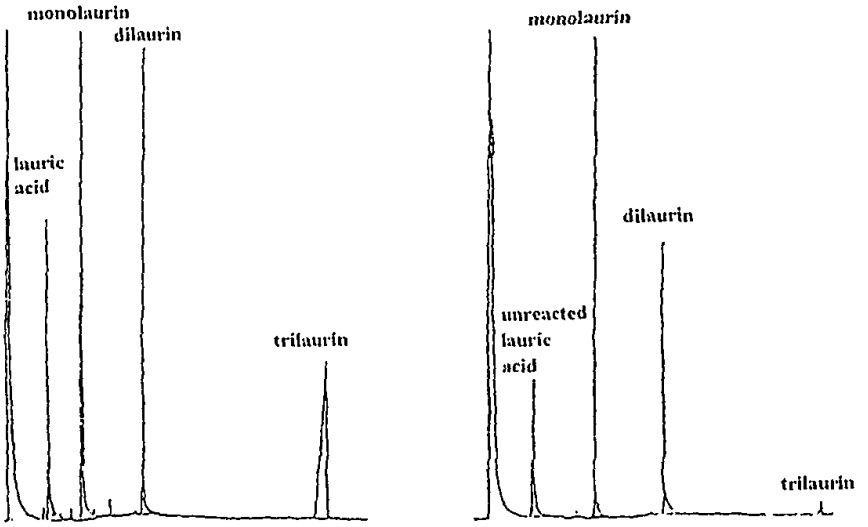



ITDI Monolaurin

## Gas Chromatograph (GC)

The compositions of the product obtained from the esterification and the unreacted fatty acid were determined using a Shimadzu GC-14 gas chromatograph. One microliter sample and the standard were injected into OV-1 capillary column at a programmed temperature of 180°C to 230°C. Through Flame Ionization Detector (FID), the components of the sample mixture and of the standard were detected with high purity nitrogen as the carrier gas. Comparative GC chromatogram of both the standard and ITDI monolaurin are shown in Figure 4.

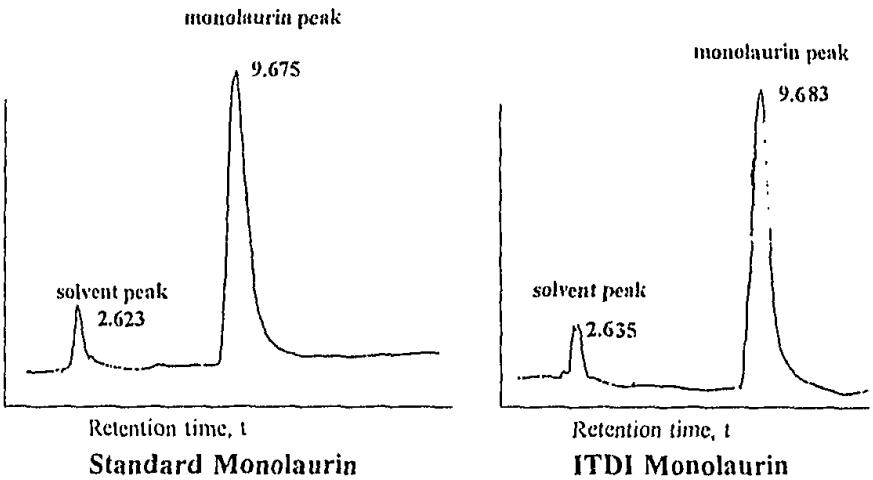
**Figure 4. Comparative GC chromatograms of standard mixture and ITDI crude monolaurin.**




## High Pressure Liquid Chromatograph (HPLC)

A 3.0% purified monolaurin in eluent solution composed of heptane: isopropyl alcohol: acetonitrile,( 88: 10:2) was subjected to HPLC analysis using Shimadzu LC-6A to determine its percentage purity. It was characterized and compared with Sigma standard monolaurin, 99% purity. A 10 microliter sample was injected into column Lichrocart 250-4 using UV-VIS spectrophotometer detector at 210 nm @ 0.04x. Comparative HPLC chromatograms of both the standard and ITDI monolaurin are shown at Figure 5.

**Figure 5. Comparative HPLC chromatograms of the standard monolaurin and ITDI monolaurin.**



### Nuclear Magnetic Resonance (NMR)

The NMR analyses were performed on a JEOL Lambda 400 NMR spectrometer, observing $^{1}H$ at 399.65 MHz and $^{13}C$ at 100.40 MHz. NMR probe used was a 5 mm tunable probe (TH5). The solvent was benzene-$D_6$; chemical shifts, δ, were referenced to tetramethyl silane (TMS).

The following NMR experiments were performed: $^{1}H$, $^{13}C$, and 2-dimensional $^{13}C$ - $^{1}H$ heteronuclear shift correlation. The $^{1}H$ and $^{13}C$ assignments are summarized in Table 3 and presented in Figure 6. These assignments are supported by the heteronuclear correlation experiment.

**Table 3. Comparison of product obtained using different methods of purification.**

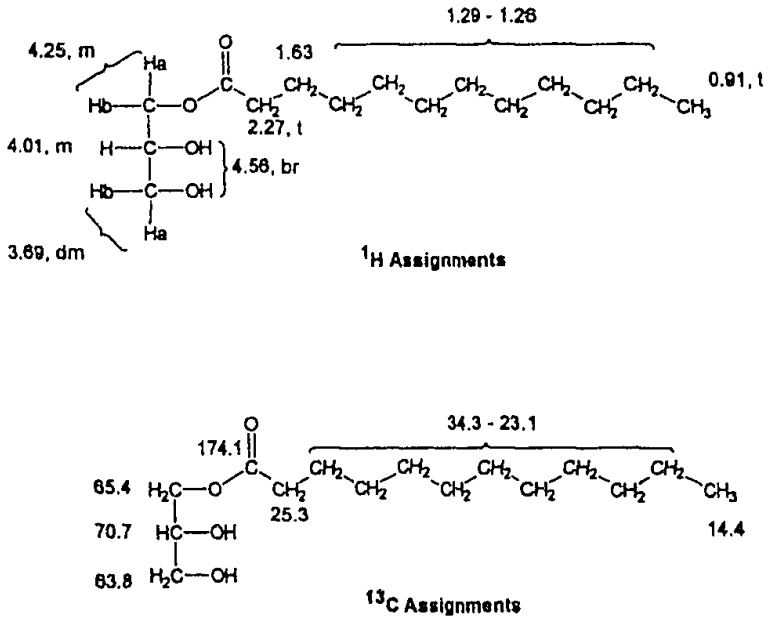
**$^{1}H$ Assignments**

| Chemicals Shift, δ | Multiplicity | Integration | Assignment |
|---|---|---|---|
| 4.56 | broad | 2 | -OH |
| 4.27 | multiplet | 2 | diastereotopic protons on C1 of glyceryl group |
| 4.01 | broad multiplet | 1 | proton on C2 of glyceryl group |
| 3.69 | doublet of multiplets | 2 | diastereotopic protons on C3 of glyceryl group |
| 2.27 | triplet | 2 | methylene protons on C2 of lauryl group |
| 1.63 | broad multiplet | 2 | methylene groups on C3 of lauryl group |
| 1.29 - 1.26 | | 16 | methylene groups on C4-C11 |
| 0.91 | triplet | 3 | terminal methyl on lauryl group |

**$^{13}C$ Assignments**

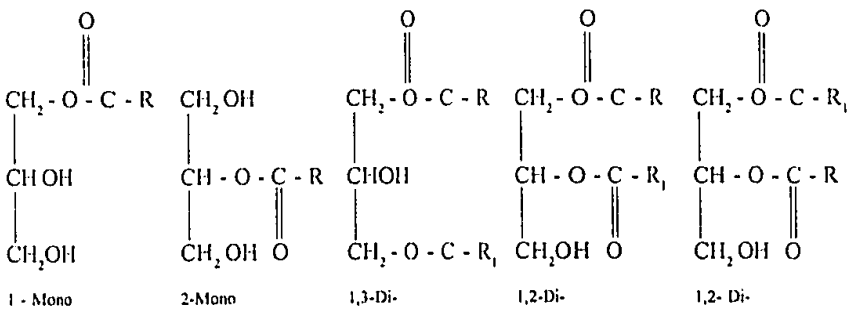
| Chemicals Shift, δ | Assignment | Chemical Shift, δ | Assignment |
|---|---|---|---|
| 174.1 | -O-CO-ester carbon | 34.3 | C2 of lauryl group |
| 70.7 | C2 of glyceryl group | 34.3 - 29.6; 23.1 | methylene carbons on lauryl group |
| 65.4 | C1 of glyceryl group | 25.3 | C2 methylene on lauryl group |
| 63.8 | C3 of glyceryl group | 14.3 | terminal methyl on lauryl group |

**Figure 6. The $^{1}H$ and $^{13}C$ assignments resulted from NMR experiments of monolaurin.**




## RESULTS AND DISCUSSION

Commercial monoglycerides usually contain only 40-60% monoester, 30-45% diester and free glycerol, depending on the ratio of fat to glycerol employed ( Swern 1979). Monoglycerides exist in several structural modifications:

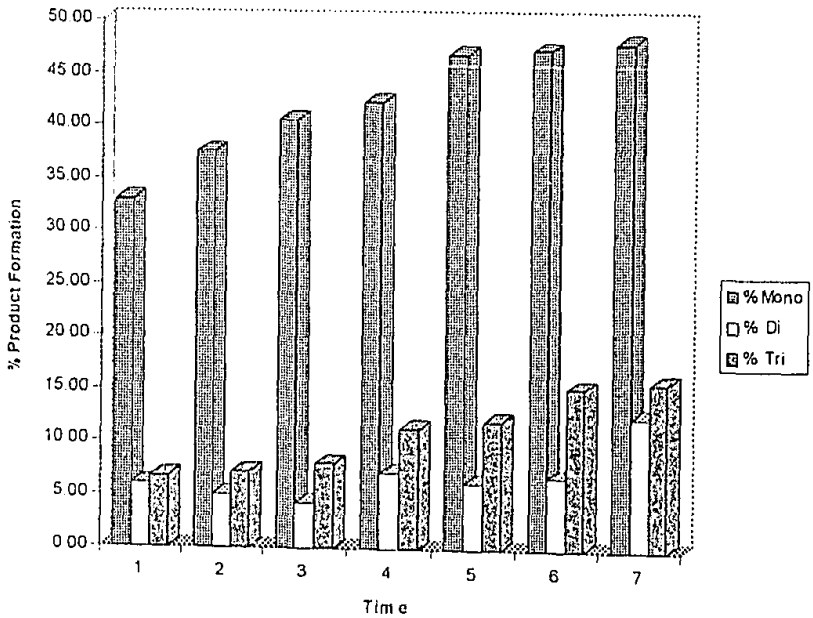



These conventional monoglycerides are usually used as preservatives, shortenings and emulsifier. With the newly found and a very potential lauric acid as an anti-microbial property, these commercial monoglycerides purity must be improved to meet the specification for food and medicine uses. To develop a high quality monoglyceride from lauric acid or monolaurin, parameter such as reaction temperature, reaction time and molar ratio were varied and studied their effects on the

product. The first parameter to be discussed is the reaction time and its effect on its formation.

Monolaurin formation was favored as time of reaction progressed up to 7th hour, as shown in Figure 7. However, while an insignificant increase in monolaurin was observed from the 5th to 7th hour, the di- and trilaurin, on the other hand, were progressively increasing during this period of reaction.

**Figure 7. Effect of reaction time on the formation of mono-, di-, and trilaurins. Conditions: Molar ratio: 1:2.5, No catalyst, Temperature: 180°C**




The effect of temperature on the formation of monolaurin is shown in Figure 8. While it is evident that monolaurin content increased with reaction temperature from 160°C to 180°C, it was also observed that the trilaurin content simultaneously increased as the temperature reaches 180°C. Beyond this temperature, it was observed that the reaction favored the formation of trilaurin even if glycerol is present in excess.

Development of the nature of esterification catalysts to be used has been directed towards those which are easily removed in the purification process. Among the non-corrosive metallic catalysts used are CaO, nickel and zinc.

Esterification done in the absence and presence of catalyst was compared. The presence of catalyst made the product darker in color. Monolaurin formation attained optimal yield in 5 hours of reaction, as shown in Figure 9.

Figure 8. Effect of Temperature on the Formation of Mono-, Di- and Trilaurins. Conditions: Reaction time: 5 hours; Molar ratio: 1:4, No catalyst.

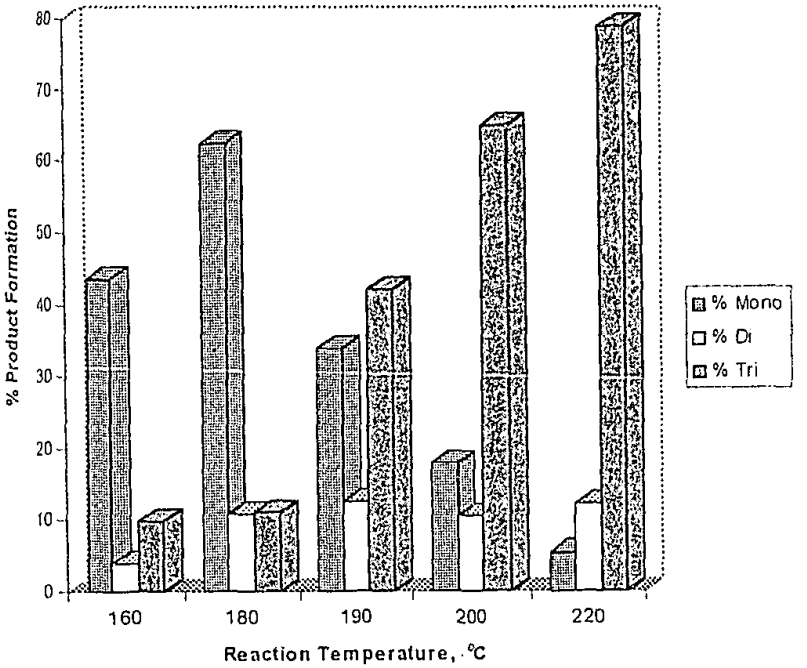
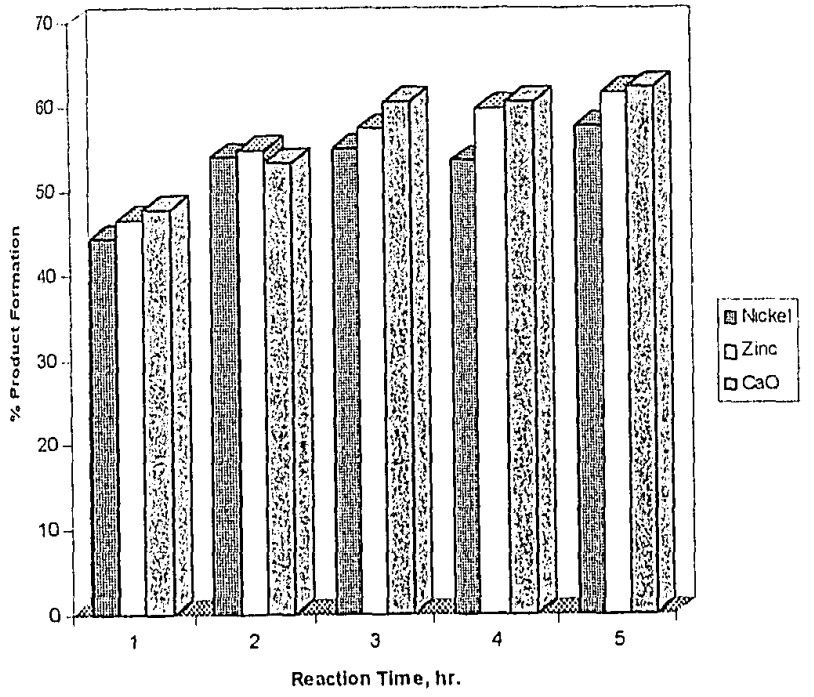
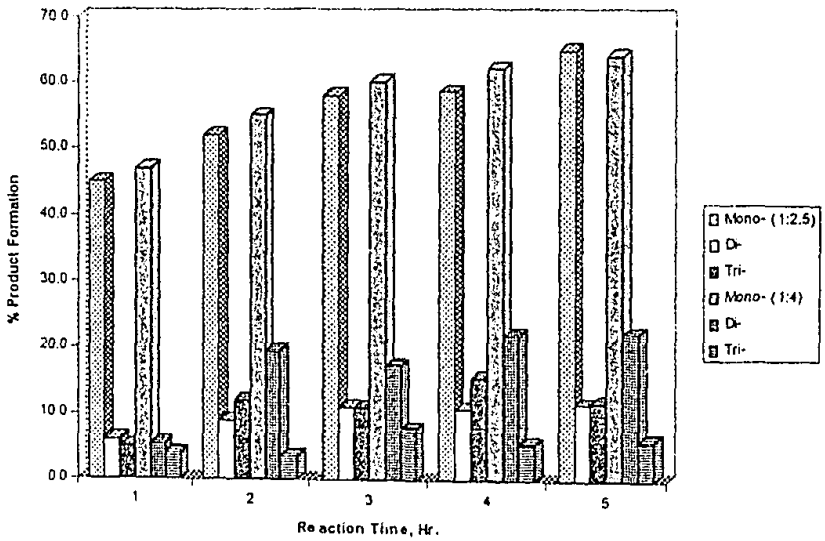



Figure 9. Effect of catalysts on the formation of mono-, di-, and trilaurins. Conditions: Reaction temp.: 180°C; Molar ratio: 1:2.5.



Another reaction parameter which was investigated is the molar ratio of the reactants. In order to obtain a product consisting mainly of monoglycerides, glycerol should be present in excess (Viernes *et al* 1990). Although several molar ratios were tried, 1:2.5 and 1:4.0 gave the most promising results. A 1:4.0 molar ratio gave almost the same amount of monolaurin product after 5 hours of reaction as shown in Figure 10. Increasing the glycerol content by 60% did not increase the monolaurin production significantly. For the economic reason , 1:2.5 molar ratio was used in this study.

**Figure 10. Effect of varying the molar ratio of reactants in the formation of mono-, di-, and trilaurins. Conditions: Reaction Temp: 180°C; No catalyst.**




## CONCLUSION

A process has been developed for the preparation of a low volume but high value monolaurin from coconut $C_{12}$ fatty acid. It was established that the following reaction conditions would be best to produce monolaurin in good yield and quality: 55.2% yield; 98.50% purity; reaction time: 5 hours; reaction temperature: 180°C; molar ratio: 1FA: 2.5 Glycerol. There were three developed purification methods for crude monolaurin namely, low temperature crystallization method, water refining method and molecular distillation method. Among the three, the latter gave the highest purity and monolaurin.

## ACKNOWLEDGEMENTS

Our thanks to Dr. Fabian Dayrit for providing the NMR results interpretation. Also, to Ms. Merle Villanueva, our Chief, Ms. Ernanda Suavillo, for her time and efforts in doing the graphs and figures, done and presented in the text, and to the rest of the staff of the Organic Chemical Section of the Chemicals and Mineral Division, who in one way or the other, have helped in the completion of this work.

We would also like to thank Cocochem Philippines for providing the $C_{12}$ fatty acid throughout the study.

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# A Bioactive Thiophene Derivative from Chrysanthemum coronarium

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## ABSTRACT

*The $CHCl_3$ extract of the air-dried leaves of Chrysanthemum coronarium afforded N-isobutyl-6-(2-thiophenyl)-2,4-hexadienamide (1). The structure of 1 was determined by extensive 1D and 2D NMR spectroscopy and high resolution mass spectrometry and comparison with published literature. Antimicrobial tests on 1 by the agar well method indicated that it has low activity against B. subtilis, P. aeruginosa, C. albicans and T. mentagrophytes and is inactive against S. aureus and E coli at a concentration of 20 μg. Micronucleus test indicated a 66.5% reduction in MPCE induced by Mitomycin C. Thus, 1 is an antimutagen.*

**Keyword Index:** *Chrysanthemum coronarium*, Compositae, tango, N-isobutyl-6-(2-thiophenyl)-2,4-hexadienamide, antimicrobial, antimutagen

## INTRODUCTION

*Chrysanthemum coronarium L.* commonly known as "Tango" belongs to the family Compositae. It is an ornamental plant and sometimes grown as a leafy vegetable in Baguio City. While the plant is not considered poisonous, its excessive consumption may result in intoxication. It is considered to be a sedative and used in conjunction with black pepper in the treatment of gonorrhoea. The bark is considered to be a purgative and used in the treatment of syphilis. The leaves are applied topically to lessen inflammation (Quisumbing, 1951). Previous studies on *C. coronarium* afforded quinic acid derivatives, which were shown to have antioxidant properties (Chuda et.al, 1996; Christopher, 1991).

*C. frutescens* and *C. brouss* afforded N-isobutyl-6-(2-thiophenyl)-2,4-hexadienamide (1) (Doskotch and Beal, 1970; Bohlmann and Zdero, 1967; Winterfeldth, 1963) which is of relevance to our present report. We now report the isolation, structure elucidation and bioassay of 1 from *C. coronarium*.

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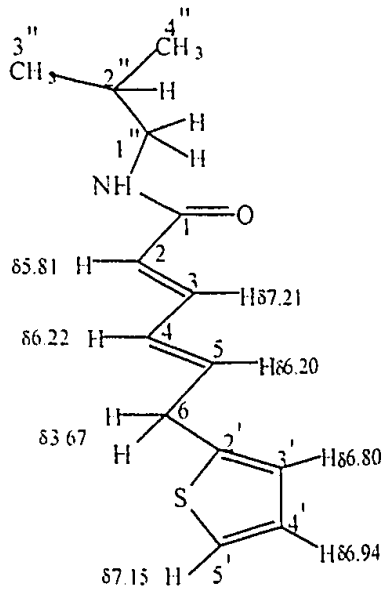
## RESULTS AND DISCUSSION

The $CHCl_3$ extract of the air-dried leaves of *Chrysanthemum coronarium L.* afforded isobutyl-6-(2-thiophenyl)-2,4-hexadienamide (**1**). High resolution electrospray ionisation MS indicated an $[M + Na]^+$ ion at 272.1072, for the molecular formula $C_{14}H_{19}NOS$. The calculated $[M + Na]^+$ value for **1** is 272.1080. The structure of **1** was determined by $^1H$, $^{13}C$, HMQC, HMBC (Table 1), COSY and NOESY NMR spectroscopy and by comparison with the $^1H$ NMR spectral data for N-isobutyl-6-(2-thiophenyl)-2.4-hexadienamide reported in the literature (Winterfeldt, 1963). This is the first structure elucidation of **1** using complete NMR spectral data.

**Table 1. 300 MHz $^1H$, $^{13}C$ and HMQC NMR spectral data of 1.**

| Carbon | $^{13}C$, δ | $^1H$, δ (HMQC) | 1H, δ (HMBC) |
|---|---|---|---|
| 1 | 166.1 | | H-3, H-2, H-1" |
| 2 | 123.4 | 5.81 (d, 14.9 Hz) | |
| 3 | 140.4 | 7.21 (m) | H-6 |
| 4 | 127.0 | 6.22 (s, br) | |
| 5 | 129.5 | 6.20 (s, br) | H-6, H-2 |
| 6 | 33.1 | 3.67 (2H, d, 4.5 Hz) | H-5 |
| 2' | 141.7 | | H-6, H-3 |
| 3' | 135.2 | 6.80 (dd, J=3.5, 1.1 Hz) | H-6, H-4', H-5' |
| 4' | 127.0 | 6.94 (dd, J=3.5, 5.1 Hz) | H-5' |
| 5' | 124.0 | 7.15 (dd, J=1.1, 5.1 Hz) | H-6, H-4' |
| 1" | 47.0 | 3.17 (2H, t, 6.6 Hz) | H-2", H-3", H-4" |
| 2" | 28.6 | 1.80 (3H, m) | H-1", H-3", H-4" |
| 3", 4" | 20.1 (2C) | 0.92 (6H, d, J=6.7 Hz) | H-1", H-2" |
| | | 5.50 (NH, s, br) | |

The COSY spectrum showed two isolated spin systems as follows. The olefinic proton at δ 7.15 (H-5') is coupled to the hydrogen at δ 6.94 (H-4'), which is in turn coupled to the proton at δ 6.80 (H-3'). The latter hydrogen is allylically coupled to the methylene protons at δ 3.67 (H-6), which are coupled to the olefinic proton at δ 6.20 (H-5), which is in turn coupled to another olefinic hydrogen at δ 6.22 (H-4), which is further coupled to the olefinic proton at δ 7.21 (H-3), which is finally coupled to the olefinic hydrogen at δ 5.81 (H-2). Another isolated spin system is

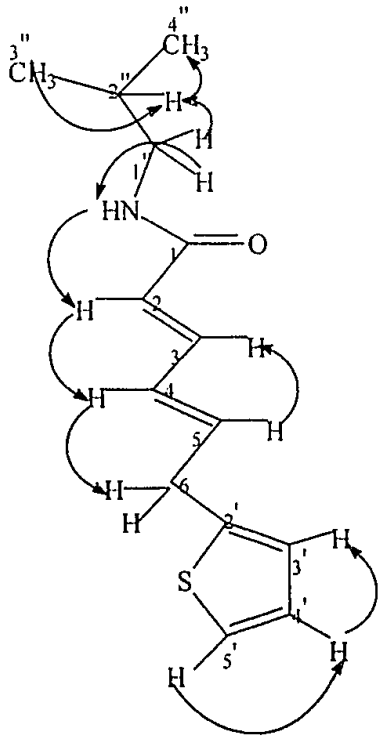



1

shown by the methyl groups at δ 0.92 (H-3", H-4") which are coupled to the methine proton at δ 1.80 (H-2"), in turn coupled to the methylene hydrogens at δ 3.17 (H-1") and finally coupled to the proton attached to a nitrogen at δ 5.50.

The relative stereochemistry of **1** was deduced from NOESY (Fig. 1). The olefinic proton at δ 7.15 (H-5') is close in space to the hydrogen at δ 6.94 (H-4'), which is in turn close to the proton at δ 6.80 (H-3). The latter hydrogen is close to the methylene protons at δ 3.67 (H-6), which are close to the olefinic proton at δ 6.20 (H-5), in turn close in space to another olefinic hydrogen at δ 6.22 (H-4), which is close to the olefinic proton at δ 7.21 (H-3) and finally close to the olefinic hydrogen at δ 5.81 (H-2). The methyl groups at δ 0.92 (H-3", H-4") are close in space to the methine proton at δ 1.80 (H-2"), which is in turn close to the methylene

**Figure 1. NOESY correlations observed for 1.**




hydrogens at δ 3.17 (H-1"), which are finally close to the proton attached to a nitrogen at δ 5.5 (N-H).

To test for possible biological activity of **1**, antimicrobial and antimutagenicity tests were conducted.

The antimicrobial tests on **1** (Table 2) at a concentration of 20 μg by the agar well method indicated that it inhibited the growth of *B. subtilis*, *P. aeruginosa*, *C. albicans* and *T. mentagrophytes* with an antimicrobial index (AI) of 0.2, but was found inactive against *S. aureus* and *E coli*. An

Table 2. Antimicrobial tests results*.

| Sample | Concn. (μg) | *Staphylococcus aureus* | | *Escherichia coli* | | *Bacillus subtilis* | | *Candida albicans* | | *Trichophyton mentagrophytes* | | *Pseudomonas aeruginosa* | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | C.Z (mm) | A.I (mm) | C.Z (mm) | A.I (mm) | C.Z (mm) | A.I (mm) | C.Z (mm) | A.I (mm) | C.Z (mm) | A.I (mm) | C.Z (mm) | A.I (mm) |
| 1 | 10 | ----- | 0 | ----- | 0 | ----- | 0 | 12 | 0.2 | ----- | 0 | 12 | 0.2 |
| | 20 | ----- | 0 | ----- | 0 | 12 | 0.2 | 12 | 0.2 | 12 | 0.2 | 12 | 0.2 |
| Standard | 20 | 22 | 1.2 | 22 | 1.2 | 22 | 1.2 | 28 | 1.8 | 50 | 4.0 | 20 | 1.0 |
| Antibiotic | | Chloramphenicol | | Tetracycline | | Chloramphenicol | | Clotrimazole | | Clotrimazole | | Tetracycline | |

*Conducted at UP-NSRI.

AI of 0.2 is much lower than the AI of standard antibiotics at the same concentration of 20 μg. Thus, **1** has low antimicrobial activity.

The antimutagenicity potential of **1** was determined by the Micronucleus test. Results of the study (Table 3) indicated that **1** at a dosage of 8 mg/kg reduced the number of micronucleated polychromatic erythrocytes (MPCE)

**Table 3. Effect of 1 on the formation of micronucleated polychromatic erythrocytes.**

| Sample | Dosage (mg/kg) | No. of MPCE/1000PCE±σ* | % Reduction in MPCE |
|---|---|---|---|
| 1 | 8.0 | 2.93 ± 1.12 | 66.5 |
| Mitomycin C | 2.75 | 8.75 ± 2.18 | |
| DMSO | 7.5 mL/kg | | |

*Determined from 15 slides

induced by mitomycin C by 66.5%. Statistical analysis using the t-test showed that there is a significant decrease in MPCE at $\alpha = 0.01$. Thus, **1** is an antimutagen.

## EXPERIMENTAL

### General Experimental Procedures

NMR spectra were recorded in $CDCl_3$ solutions on a Bruker AMX 300 NMR spectrometer with $CDCl_3$ ($\delta$ 7.26, 77.0 ppm) as reference. The high resolution ESIMS were recorded on a Bruker BioApex 47e (FTMS) mass spectrometer. Silica gel type 60 (Merck) was used for column chromatography and plastic-backed plates coated with Silica gel F254 (Merck) for thin layer chromatography. Plates were visualized by spraying with vanillin/$H_2SO_4$ and warming.

### Sample Collection and Isolation

The plant sample was collected from the Teacher's Camp, Baguio City in September 1995. It was identified as *Chrysanthemum coronarium L.* at the Philippine National Museum and a voucher specimen is kept at the Chemistry Department of De La Salle University.

Air-dried leaves (300 g) of *C. coronarium* L. were extracted with $CHCl_3$ to afford a crude extract (20 g) which was treated with aqueous lead acetate to precipitate the pigments (Padolina et.al, 1974). The treated extract was subjected to gravity column chromatography (dry packing) with the use of increasing proportions of $Me_2CO$ in $CHCl_3$ (10% increment) as eluents. The 20% $Me_2CO$ in $CHCl_3$ fraction was rechromatographed in 10% $Me_2CO$ in $CHCl_3$ to afford **1** (15 mg, 103-105 °C).

## Bioassay

### 1. Antimicrobial Tests

The microorganisms used in these tests are *Staphylococcus aureus* UPCC 143, *Bacillus subtilis* UPCC 1, *Escherichia coli* UPCC 195, *Pseudomonas aeruginosa* UPCC 244, *Candida albicans* UPCC 2168 and *Trichophyton mentagrophytes* UPCC 4193.

A microbial suspension containing approximately $10^7$ cells/mL was prepared for each test organism for 24-hour agar culture using 0.1% peptone water. One-tenth (0.1 mL) of the bacterial suspension was transferred into pre-poured 30 mL deep nutrient agar plate, the yeast suspension into glucose yeast peptone agar plate and the fungal suspension on potato dextrose agar plate. About 5 mL of the corresponding melted agar cooled to about 45 °C was immediately poured into the plate. The plate was swirled to distribute the microbial cells evenly on the plate. After the overlay agar has solidified, three 1-cm diameter holes were bored from equidistant points using a sterile cork borer.

One-tenth (0.1 mL) portions of the extract were placed in duplicate holes per organism. A similar volume of the solvent acetone and of the corresponding antibiotic for each test organism was placed in the remaining two wells on the plate. Plates were incubated at room temperature to prevent evaporation of liquid on the petri lid that may cause interference in distribution of organisms on the surface. Bacterial and yeast plates were read after 24 hours, while the mold plate was read after three days. Clearing zones were measured in millimeters (mm). The average for each supernatant was taken and the antimicrobial activity index (AI) was computed.

### 2. Antimutagenicity Test

The test compounds (8 mg/kg mouse) dissolved in DMSO (7.5 mL/kg mouse, solvent control) were administered simultaneously with mitomycin C (2.75 mg/kg mouse, positive control) to mice of the Swiss strain. For the control, only mitomycin C and DMSO were administered simultaneously to the mice. Five mice were tested for each compound and control. The second administration was carried out after twenty-four hours. Six hours after the second administration, the mice were killed by dislocation of the neck. Blood from the bone marrow was smeared on slides (three per mouse). The slides were stained with May Grunwald and Giemsa solutions (Schmid, 1972). The numbers of MPCE/1000 PCE were counted by the use of a high power microscope.

## ACKNOWLEDGMENT

The authors would like to acknowledge Dr. David Bourne of the Australian Institute of Marine Science for the high resolution mass spectrum of the thiophene derivative. The antimicrobial tests were conducted at the University of the Philippines-Natural Science Research Institute (UP-NSRI).

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# Ipil-Ipil Seed Gum: Part I. Isolation and Characterization

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## ABSTRACT

*The crude gum from the seeds of Leucaena leucocephala (Lam.) de Wit was purified by fractional precipitation with ethanol followed by ion exchange chromatography using DEAE-cellulose column. Physicochemical and chemical studies showed that the protein-free gum exhibited properties similar to that of galactomannan.*

## INTRODUCTION

Gums are a group of industrially useful polysaccharides. Natural gums are used in the pharmaceutical, food, paper, oil and gas, mining, textile, and explosives industries.

A locally grown plant that can be tapped for gum production is the leguminose *Leucaena leucocephala* (Lam.) de Wit, locally known as ipil-ipil. Ipil-ipil is found throughout the Philippines and produces seeds in great abundance (Quisumbing, 1978). The proximate chemical composition of seven varieties of ipil-ipil seeds were found to be 6.52%-10.89% moisture, 2.60%-3.82% ash, 3.95%-13.76% crude fat, 8.25%-11.08% crude fiber, 19.74%-34.14% crude protein, 3.16%-5.04% total nitrogen, 37.44%-49.45% nitrogen-free extract, and 48.03%-60.63% total carbohydrates (Pamplona, 1986; Hibec, 1979; Espiritu, 1977). Ipil-ipil seed is a potential source of gum because of its high carbohydrate content.

A recent study on ipil-ipil seed gum showed it can be used in the paper industry as a paper making additive (Pamplona, 1986). However, for the food and pharmaceutical industries, the ethanol precipitated gum has to be further purified. The presence of mimosine, the toxic amino acid analogue, has prevented the use of the gum. This study focuses on the isolation and characterization of a protein-free *L. leucocephala* seed gum.

## METHODOLOGY

### Isolation of the Seed Gum

Ipil-Ipil seeds of the K-8 variety (Hawaiian giants) were obtained from the Manila Seedling Foundation. Preliminary scarification procedures included: (a) hot water treatment; (b) ordinary water treatment; and (c)

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acid treatment. However, these pre-treatments did not ease decoating oven-dried seeds by hand.

Ipil-ipil seeds were oven dried at 40°C for 30 minutes prior to decoating and subsequent grinding. The powdered seeds were then immersed in petroleum ether to extract the fat soluble matter. The sample was mixed using a VWR vortexer 2 (Bohemia, New York). After centrifugation for 10 mins at 2500 x g using an IEC HN-SH centrifuge (Needham, Mass.), the petroleum ether layer was siphoned off. The remaining traces of ether was evaporated by heating in a water bath.

The fat free sample was then immersed in distilled water. The sample was vigorously stirred for 1 hr on a Corning stirring plate, followed by sonication for 2 hrs in a Bransonic tank sonicator. The sample was then centrifuged for 10 min at 2500 x g. The supernatant was decanted into a clean beaker. The extraction process with distilled water was repeated until a ratio of 1 kg seeds: 1 liter $H_2O$ was obtained. The aqueous layers were then combined.

Ethanol was added slowly to the water extract, with rapid stirring, until an alcohol concentration of 45% by volume was obtained. The precipitated gum was then recovered by centrifugation. The supernatant was discarded. The residue was washed with ethanol and then dried inside an oven at 40°C for 1 hr.

The crude gum was purified by fractional precipitation as follows: a 0.8% aqueous suspension of the crude gum was autoclaved at 15 psi for 3 hrs at pH 5.8 and immediately centrifuged for 10 min at 2500 x g. The insoluble components were discarded. Sufficient ethanol was then added to the aqueous solution to bring the alcohol concentration to 25% by volume. The resulting precipitate was recovered and subjected to quantitative protein analysis.

More ethanol was slowly added to the aqueous solution, with rapid stirring, to increase the alcohol concentration to 40% by volume. The precipitated gum was then recovered by centrifugation for 10 min at 2500x g, after which it was washed with absolute ethanol and oven-dried at 40°C for 1 hr. The protein content of the gum powder was then quantitatively determined by the Kjeldahl method.

### Purification of the Crude Gum

An ice cold aqueous solution was then prepared by dissolving the ethanol-precipitated gums (10mg) in 4 ml distilled water. Cold trichloroacetic acid (TCA) was then added to a final acid concentration of 5% by volume (Lesniak and Liu, 1981). The gum remained in solution in the TCA while impurities were precipitated. The residue was separated by centrifugation for 10 min at 2500 x g and discarded. The TCA soluble sample was applied to a 1 cm I.D. x 63 cm long column containing diethylaminoethyl

(DEAE)-cellulose anion exchange resin. Elution was performed with a 5% TCA solution. Five ml fractions were collected. The TCA was then converted to perchloroester by addition of methanol; this was removed by distillation under reduced pressure. Ethanol was added to the remaining sample to a concentration of 40%. The precipitate was recovered by centrifugation for 10 min at 2500 x g, washed with absolute ethanol and freeze-dried.

### Characterization Of The Pure Seed Gum

The physical properties of the pure gum such as form, color, odour, pH and viscosity of a 1% aqueous solution were observed. The viscosity of the gum was measured using a Brookfield rotational viscometer which uses a RV2 spindle immersed in the test specimen.

Several identification tests were performed on the pure seed gum: specific reaction with $Ba(OH)_2$ (Glicksman, 1969); water solubility properties; alcohol precipitability characteristics; and microscopic identification. For the confirmatory test as a galactomannan, 1 ml of 4% borax was added to 3-5 ml of the gum solution.

The coagulability of the gum was determined quantitatively by measuring the milliequivalents of reagent required to produce coagulation or flocculation of 1 g of the gum. The following precipitating reagents were used: tannic acid, Fehling's solution, lead acetate, borax, and phosphotungstic acid (Glicksman, 1969).

Also noted were the characteristic reactions of the gum with specific reagents: Millon's, neutral lead acetate, basic lead acetate, KOH, $FeCl_3$, Schweitzer's reagent, Stoke's acid, mercuric nitrate, sulfuric acid, aluminum chloride, and saturated ammonium sulfate (Glicksman, 1969).

The infrared spectrum of the gum was determined in KBr pellets from 4000 to 200 $cm^{-1}$ in an Analect RFX-40 FTIR Spectrophotometer. Characteristic peaks were found at 3400 (br), 2900, 1650, 1400, 1160-1100 $cm^{-1}$.

### Chemical assay of ipil-ipil seed gum

Qualitative tests such as Benedict's, iodine test, and Biuret test were performed on the seed gum to determine the presence of reducing sugars, starch, and proteins, respectively.

The following quantitative tests: %moisture, %crude fiber, %acid insoluble residue, %total ash, %protein, and %starch were determined according to NF XVII and USP XXII (1989). The percent gum was calculated by subtracting from 100 the sum of the chemical components which were identified and quantified.

### Test for total ash

Exactly 2 g seed gum was incinerated in a tared crucible at a low temperature not to exceed dull redness, until free from carbon. The ash was then weighed. If a carbon-free ash is not obtained this way, the charred mass was boiled with hot water. The insoluble residue was collected on an ashless filter paper and was incinerated until the ash becomes white or nearly so. The filtrate was then added and evaporated to dryness. The sample was then heated to a dull redness. If a carbon-free ash is still not obtained this way, the crucible was cooled. To it was added 15 ml of the alcohol. The ash was broken with a glass rod and the alcohol burned off. The whole was again heated to a dull redness. The sample was cooled, weighed, and the percentage total ash calculated.

### Test for acid-insoluble matter

1.5 g seed gum was transferred to a 250 ml beaker containing 150 ml of water and 1.5 ml conc. $H_2SO_4$. The beaker was covered with a watch glass, and the mixture was heated on a steam bath for 6 hrs. Any water lost by evaporation was replaced. At the end of the 6-hr heating period, about 5500 mg of a suitable filter aid was added and filtered through a tared filtering crucible provided with an asbestos pad or through a suitable ashless filter. The residue was washed several times with hot water until the filtrate was free of acid. The filter with its contents was dried at 105°C for 3 hrs, then cooled in a dessicator and weighed. The amount of solid insoluble matter was determined by subtracting the weight of the filter aid from that of the residue.

### Test for crude fiber

About 2 g of the gum was thoroughly mixed with ether. After removal of the ether by evaporation in a steam bath, 200 ml of boiling dilute $H_2SO_4$ (1 in 78) was added to the gum sample in a 500 ml flask. The mixture was then refluxed for exactly 30 min. Then it was filtered through a hardened-paper filter. The residue on the filter was then washed with boiling water until pH > 7. The residue was rinsed back into the flask with 200 ml of boiling 1.25% NaOH solution. The mixture was again refluxed for exactly 30 min, then rapidly filtered through a tared filter. The residue was washed with boiling water until the last washing becomes neutral. It was then dried to constant weight at 110°C. After cooling in a dessicator, the ash was weighed. The difference between the weight obtained by drying at 110°C and that of the ash represents the weight of the crude fiber.

### Test for moisture

Moisture content was determined using the Karl Fischer titration method recommended by the United States Pharmacopeia (USP XXII, 1619-1621).

### Test for proteins

A 2.0 g sample of the gum was transferred to a 500 ml Kjeldahl flask, followed by addition of 25 ml conc. $H_2SO_4$, 7 g $K_2SO_4$ and 0.70 g HgO. The mixture was boiled in a hot plate until the liquid becomes colorless. This was continued for 10 to 15 min. After cooling, the funnel and neck were rinsed with 75-100 ml distilled water. More distilled water was added to make a total of 200 ml solution. The sample was then cooled to room temperature and 40 ml of 50% NaOH was added. 25 ml of 0.1N HCl was transferred to an Erlenmeyer flask and 5 to 10 drops of phenolphthalein TS was added. The Kjehldahl flask was connected to a distillation unit with the Erlenmeyer flask as the receiver. The mixture was heated until all the $NH_3$ distills over. The excess acid in the Erlenmeyer flask was titrated back with 0.1 N NaOH. Percentage nitrogen was calculated and multiplied by the factor 6.25 to give percentage protein.

## RESULTS AND DISCUSSION

From the aqueous extract of the fat free ipil-ipil seeds, 17% of the original weight was precipitated out by repeated treatment with ethanol. The lyophilized product was a fluffy, tan colored powder with a beany odor. Table 1 shows the chemical components of the crude ipil-ipil seed gum.

**Table 1. Chemical assay of L. leucocephala seed gum.**

| Component | % Assay | |
|---|---|---|
| | Crude Ipil-Ipil Seed Gum | Pure Ipil-Ipil Seed Gum |
| Acid Insoluble Residue | 3.1 | 4.0 |
| Protein | 8.2 | negative |
| Starch | negative | negative |
| Moisture | 10.2 | 10.0 |
| Crude Fiber | 5.9 | 5.8 |
| Total Ash | 1.8 | 1.3 |
| Gum Content | 70.8 | 78.9 |

% gum was calculated by subtracting from 100 the sum of the other components.

The preliminary purification of the seed gum by fractional precipitation with ethanol yielded a light brown powder with 85% recovery (Table 2) and a decrease in protein content to 4%. Addition of cold trichloroacetic acid (TCA) to the gum precipitated impurities while the gum remained in solution. It was earlier reported that addition of TCA removes virtually all mimosine (Lesniak and Liu, 1981). Further purification by passing the seed gum through a DEAE-cellulose column, yielded a cream colored powder with 76% recovery. A negative Biuret test proves that proteins in the crude gum have been removed.

**Table 2. Data of purification method of crude L. leucocephala gum.**

| Method Applied | % yield[a] | Biuret[b] | Benedict's[c] | Kjeldah[d] |
|---|---|---|---|---|
| Alcohol precipitation | 17.0 | | | |
| Fractional precipitation | 14.45 | positive | positive | 4% |
| Ion exchange chromatography | 12.92 | negative | negative | * |

[a]based on original weight of seeds
[b]test for proteins
[c]test for reducing sugars
[d]test for proteins
*below sensitivity limit

The addition of saturated $Ba(OH)_2$ to the ipil-ipil gum solution produced immediate precipitation. The precipitate was voluminous, opaque and stringy. This implies the presence of glucomannans, galactoglucomannans, and galactomannans (Glicksman, 1969). A colloidal solution was formed when the gum was dissolved in cold water. The solution was viscous and opalescent. When the gum solution was heated in a water bath for 10 mins, no appreciable increase in viscosity was produced. The alcohol precipitability characteristic test showed that the alcohol precipitate was stringy, clotty, opaque, and non-adherent.

The ipil-ipil seed gum formed a gel upon addition of 4% borax, which is a very clear indication of the presence of a galactomannan. It is known that all galactomannans produce gels or mucilaginous precipitates when boric acid is added to their aqueous solutions. This reaction is general with polysaccharides containing adjacent hydroxyl groups in the cis position and is due to the extensive cross-linking produced (Stein, Hall & Co., Inc., 1962).

Table 3 presents the results of the various physical tests performed on the pure seed gum. The results of the chemical assay on the pure gum are tabulated in Table 1. The coagulability of the pure gum with various electrolyte reagents is in Table 4, where the milliequivalents of reagent required to produce coagulation or flocculation of 1 g of the gum is reported. The identification of the pure gum by its characteristic reactions with specific reagents is presented in Table 5.

**Table 3. Physical properties of pure *L. leucocephala* seed gum.**

| Physical Property | L. leucocephala Gum |
|---|---|
| Appearance (Form) | powder |
| Color | cream |
| Odor/Taste | slight |
| pH of 1% aqueous solution | 5.99 |
| Viscosity | 2.43 cp |

**Table 4. Reactivity of the pure L. leucocephala seed gum *with electrolyte reagents.**

| Reagent | Flocculation value@ |
|---|---|
| Tannic Acid | 1.650 |
| Fehling's Solution | 0.120 |
| Lead Acetate | 19.00 |
| Borax | 50.00 |
| Phosphotungstic Acid | 0.500 |

0.5% concentration.
@ milliequivalents of reagent per gram of gum

**Table 5. Characteristic reaction of pure *L. leucocephala* seed gum.**

| Reagent | Reaction |
|---|---|
| Millon's reagent | gel |
| 20% lead acetate | voluminous precipitate; gels |
| basic lead acetate | opaque gel |
| 10% potassium hydroxide | slight flocculent precipitate |
| 5% ferric chloride | stringy precipitate |
| 10% tannic acid | precipitate |
| conc. sulfuric acid | precipitate |
| saturated ammonium sulfate | voluminous precipitate |

test solution is 0.5% aqueous solution of L. leucocephala gum

Since most gums have similar functional groups, little variation is shown by their infrared spectra in the shorter wavelength regions. The 7-15μ region had been used to fingerprint the seed gums such as guar gum and locust bean gum into one group. The most prominent spectral feature of these two gums is the presence of 2 strong bands at 11.55 μ and 12.35 μ separated by a trough. Noteworthy differences are also apparent in the 8.5 - 11 μ region. These spectral features had been observed with the *L. leucocephala* seed gum.

## CONCLUSION

Natural gums were isolated from ipil-ipil seeds by precipitation with ethanol of the aqueous extract followed by ion-exchange chromatography using a DEAE-cellulose column. This permitted quantitative recovery of a protein-free galactomannan in a chemically undegraded form.

## ACKNOWLEDGEMENT

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# Ipil-Ipil Seed Gum: Part II. Its Use in the Pharmaceutical Industry

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## ABSTRACT

*Physicochemical and chemical studies showed that the protein-free ipil-ipil seed gum exhibited properties similar to that of galactomannan from guar gum. A hardness test performed on tablets formulated with the purified ipil-ipil seed gum showed that it is an effective binder. A feasibility study conducted by the Asia-Pacific Centre for Research (1994) for the Philippine Council for Health Research and Development (PCHRD) showed that the ipil-ipil seed gum project is viable.*

## INTRODUCTION

A drug product is a formulation of one or more active ingredients combined with various components known as placebo. The placebo or excipients are useful in the manufacture and compounding of various pharmaceutical preparations (Peczon, 1988). They provide compressibility, solubility in gastric juice, bioavailability requirements, packageability and stability to tablets. To capsules, they add flow characteristics. To liquid formulations, they establish the pH, viscosity, and clarity properties. In effect, placebos are often referred to as pharmaceutical necessities that function as antioxidants, preservatives, colouring, flavouring, diluting, emulsifying, suspending and gelating agents, ointment bases, pharmaceutical solvents and miscellaneous agents (Gennaro, 1990).

A material that can serve various purposes of the placebo is guar gum. Guar gum is used in pharmaceuticals as a thickening agent for jelly formulations, suspensions, emulsions, lotions, creams and toothpaste; and as a binding and disintegrating agent in tablet formulation. Currently, most of the excipients used in the pharmaceutical industry are imported. In 1993, the Philippines imported a total of 278,262 kg of guar gum (Feasibility Study on Ipil-Ipil Seed Gum, 1994). The estimated gum consumption of the pharmaceutical industry is 156,740 kg/year.

This research is on the feasibility of using ipil-ipil seed gum for the pharmaceutical industry, specifically as a substitute for guar gum. This is in line with the objective of the Philippine drug industry to develop

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affordable but effective drugs based on our indigenous plant resources (Dayrit, 1988). Development of locally produced gums will greatly supplement this goal.

## METHODOLOGY

### Isolation and Purification of the Seed Gum

Ipil-Ipil seeds of the K-8 variety (Hawaiian giants) were obtained from the Manila Seedling Foundation. The ipil-ipil seed gum was extracted with water and precipitated with ethanol as described in Faustorilla et. al., Part I. The crude seed gum was purified by fractional precipitation with ethanol followed by ion exchange chromatography using DEAE-cellulose column.

### Comparison of the Physical Properties of Ipil-Ipil Seed Gum and Guar Gum

The physical properties of the ipil-ipil seed gum and guar gum such as form, colour, odour, pH and viscosity of a 1% aqueous solution were observed.

### Comparison of the Chemical Properties of Ipil-Ipil Seed Gum and Guar Gum

The chemical properties of ipil-ipil seed gum and guar gum were also determined. Moisture, crude fiber, acid insoluble residue, total ash, protein, and starch were determined according to NF XVII and USP XXII (1989) . Percent gum was calculated by subtracting from 100 the sum of the chemical components which were identified and quantified.

### Hardness Test

The binding effect of *L. leucocephala* seed gum was determined by following the tablet formulation for acetylsalicylic acid obtained from Remington's Pharmaceutical Sciences (Gennaro, 1990).

The raw materials (weight equivalent to 50 tablets) were blended in a mechanical mixer for 15 mins. The sample was compressed using BB3B tabletting machine (Manesty Machine). The effect of ipil-ipil seed gum and guar gum on the cohesive qualities of the tablet prepared by the dry granulation method was determined by replacing starch in the formulation. The comparative effect of ipil-ipil seed gum and guar gum on the tablet was checked by testing tablet hardness with a Stokes tester.

## RESULTS AND DISCUSSION

Physicochemical and chemical studies showed that the protein-free ipil-ipil gums exhibited properties similar to galactomannan (Faustorilla et. al., Part I). Galactomannans are branched polysaccharides commonly found in the endosperm of most legume seeds. Galactomannans are most abundant in

the seeds of guar and locust trees.

Guar seed gum is the most completely investigated galactomannan (Glicksman, 1969). It is extracted from the endosperm of the seed of *Cyamopsis tetragonolobus* and is mainly cultivated in India and Pakistan. It is used as a thickening agent for jelly formulations, suspensions, emulsions, lotions, creams, and toothpaste; and as a binding and disintegrating agent in tablets. Aside from pharmaceuticals, guar gum is also used in the food processing and paper industries.

Comparison of the physical properties of protein-free ipil-ipil seed gum and guar gum (Table 1) shows that they are both cream coloured powders

**Table 1. Physical properties of pure *L. leucocephala* seed gum and guar gum.**

| Physical Property | *L. leucocephala* Gum | Guar Gum |
|---|---|---|
| Appearance (Form) | powder | powder |
| Colour | cream | cream |
| Odor/Taste | slight beany odor | slight beany odor |
| pH of 1% aqueous solution | 5.99 | 6.04 |
| Viscosity | 2.43 cp | 2.63 cp |

with a slight beany odor. Their pH are both approximately equal to 6. Guar gum is more viscous than ipil-ipil seed gum.

The minimum chemical analyses needed to monitor the purity of commercial gums are moisture, protein, and acid-insoluble residue (Davidson, 1980) with maximum values of 15%, 10%, and 7%, respectively (NF XVII). Table 2 summarises a comparison of the chemical assays for ipil-ipil seed gum and guar gum. Both gums have similar % acid insoluble residue (≈ 4%), % moisture (≈10%), % crude

**Table 2. Chemical assay of *L. leucocephala* seed gum and guar gum.**

| Component | Ipil-Ipil Seed Gum | Guar Gum |
|---|---|---|
| Acid Insoluble Residue | 4.0% | 4.1% |
| Protein | negative | 6.0% |
| Starch | negative | negative |
| Moisture | 10.0% | 10.3% |
| Crude Fiber | 5.8% | 5.7% |
| Total Ash | 1.3% | 1.6% |
| Gum Content | 78.9% | 72.3% |

% gum was calculated by subtracting from 100 the sum of the other components.

fiber (≈ 5.7%), and % total ash (≈ 1.5%). They both tested negative for starch. They differ in protein content with guar gum containing 6% proteins.

The tablet is the most frequently employed dosage form (Finch, 1983) as it offers precision of dosage and convenience of administration. Binders are agents used to impart cohesive qualities to the powdered material (Lachman and Lieberman, 1980). To determine the binding capacity of ipil-ipil seed gum, acetylsalicylic acid (aspirin) tablets were formulated

**Table 3. Composition and hardness of various batches of aspirin tablets.**

| Batch No. | % Binding agent* | Table Hardness | |
|---|---|---|---|
| | | *L. leucocephala* gum | guar gum |
| 1 | 5 | 6.2 | 6.1 |
| 2 | 10 | 6.7 | 6.5 |
| 3 | 15 | 7.0 | 7.0 |

% of tablet weight.

with ipil-ipil seed gum as the binding agent. The hardness of the tablet served as a measure of its binding action. At 5% and 10% binding agent, the binding effect of ipil-ipil seed gum was greater than that of guar gum (Table 3). These results show that ipil-ipil seed gum is a good binder.

The quantity of binder used has considerable influence on the characteristics of the compressed tablet. The use of too much binder will make a hard tablet which will not disintegrate easily. As a Stokes hardness of 6 is the normal criteria for tablet hardness, it is suggested that the ipil-ipil seed gum content in the tablet be at a minimum of 5% of the tablet weight.

A feasibility study conducted by the Asia-Pacific Centre for Research (1994) for the Philippine Council for Health Research and Development (PCHRD) showed that the ipil-ipil seed gum project is a viable project with an impressive internal rate of return of slightly above 50%. The project cost is about 3 million pesos with a sales revenue of about 10 million for year 1 alone. Positioning the project to supply gums for the pharmaceutical industry is a good entry strategy as the pharmaceutical industry alone requires about 300,000 kg of gum per year. Future research and development activities would include the use of ipil-ipil seed gum in the chewing gum, food, and paper industries.

## CONCLUSION

The physical and chemical properties of ipil-ipil seed gum showed that it exhibited properties similar to galactomannan from guar gum. Its adhesive

character shows that ipil-ipil seed gum is a good tablet binder. A pre-feasibility study conducted by the Asia-Pacific Centre for Research (1994) for the Philippine Council for Health Research and Development (PCHRD) showed that the ipil-ipil seed gum project is a viable project with an impressive internal rate of return of slightly above 50%.

## ACKNOWLEDGEMENT

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# Ipil-Ipil Seed Gum: Part III. Bulk Extraction and Mutagenicity/Antimutagenicity Studies

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## ABSTRACT

*Bulk extraction of ipil-ipil seed gum was done on coated seeds by hot percolation. The crude gum was purified by fractional precipitation. The ipil-ipil seed gum is a cream to light brown powder with a slight beany odor. Chemical analysis showed that its % protein is less than 0.3%.*

*The results of the Micronucleus Test showed that the ipil-ipil seed gum is not mutagenic at dosages of 3, 18, 35 and 50 mg / kg mouse. Kruskal-Wallis One Way Analysis of Variance by Ranks showed that the ipil-ipil seed gum at the different dosages and water (solvent control) belong to the same population at* $\alpha = 0.001$.

*The same dosages of ipil-ipil seed gum reduced by 67.3%, 53.6%, 71.8%, and 85.8%, respectively, the number of micronucleated polychromatic erythrocytes (MN-PCE) induced by tetracycline, a known mutagen. Thus the ipil-ipil seed gum is antimutagenic. Statistical analysis showed that the variances of ipil-ipil seed gum at dosages of 3, 35, and 50 mg / kg mouse is statistically different to that of tetracycline (positive control) at* $\alpha = 0.001$.

## INTRODUCTION

Protein-free ipil-ipil seed gum was isolated by cold immersion in water of decoated seeds followed by alcohol precipitation. The crude seed gum was purified by fractional precipitation and then by ion-exchange chromatography (Faustorilla et. al., Part I). Ipil-ipil seeds are normally decoated manually by using pliers to crack the seed coat because multistage grinding and sifting cannot separate the coat from its endosperm. Thus, the processes of decoating is time-consuming while purifying by ion-exchange chromatography is expensive.

A mutation of a gene is a change of one or more of the nucleotides in DNA. Consequences of such mutations include cell death; physical malformations when it attacks cells during organogenesis; cancer when it attacks somatic cells; or sterility and genetic disorders when it attacks germ cells. Hence, it is imperative that therapeutics and excipients be screened for their genotoxic effects.

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Antimutagens are substances that lower the spontaneous and induced mutation rates of the chromosomes (Sylianco, 1990) and they are classified as desmutagens or bioantimutagens (Kada *et. al.*, 1981; Sylianco and Shier, 1985).

This study on the use of a time- and cost- effective procedure in the extraction of ipil-ipil seed gum and its subsequent *in vivo* mutagenicity and antimutagenicity testing using the Micronucleus Test.

## METHODOLOGY

### Isolation and Purification of Ipil-Ipil Seed Gum

Ipil-Ipil seeds of the K-8 variety (Hawaiian giants) were obtained from the Manila Seedling Foundation. The coated ipil-ipil seeds were ground and were then placed in boiling water to disperse the gum. The solution was then filtered. The crude ipil-ipil seed gum was purified by fractional precipitation (Faustorilla et. al., Part I).

### Characterization of Ipil-Ipil Seed Gum

The physical properties of the ipil-ipil seed gum such as appearance, colour, odour, and pH of a 1% aqueous solution were observed. Moisture, crude fiber, acid insoluble residue, total ash, protein, and starch were determined according to NF XVII and USP XXII (1989). Percent gum was calculated by subtracting from 100 the sum of the chemical components which were identified and quantified.

### Mutagenicity and antimutagenicity testing using the Micronucleus Test (Schmid, 1975)

Albino mice, 7-12 weeks old, were used. Five mice were used per dosage of the purified gum, and 5 mice each were used for the positive and solvent controls. The required weights of the purified gums were dissolved in distilled water. The required weight (55 mg / kg mouse) of tetracycline (Upjohn) was dissolved in distilled water. Tetracycline was administered orally using a gavage while the purified gum was injected intraperitoneally into the test animals 30 and 6 h before the mice were sacrificed.

Six hrs after the second administration, the mice were killed by cervical dislocation. Both femora were removed by cutting through the pelvis and tibia. After removal of the muscle tissues, the distal epiphyseal portion was torn off. The proximal end of the femur was shortened carefully with scissors until a small opening to the marrow canal was seen. About 0.2 ml of fetal calf serum (Gibco) was introduced into a 1-ml syringe and then the needle was inserted a few mm into the proximal part of the marrow. The femur was then submerged completely into 2 ml of serum in a 5-ml test tube and the marrow was aspirated. After flushing and aspirating several times, the test tube was centrifuged several times and the supernatant was decanted. The cells in the sediment were mixed carefully using a Pasteur pipette and aspirator, and were then smeared on glass slides. The three slides prepared

for each were left overnight to dry.

The following procedure was used in staining the slides: 3 min in undiluted May-Grunwald (Sigma Diagnostics) stain , 2 min in 50% May Grunwald stain solution diluted with distilled water, and 10 min in 15% aqueous Giemsa (Merck) stain solution.

The number of micronucleated polychromatic erythrocytes (MN-PCE) per 1000 polychromatic erythrocytes (PCE) was counted using a high power microscope (10 X 40) (Olympus).

## RESULTS AND DISCUSSION

The ipil-ipil seed gum was obtained using the hot percolation method. The seeds were not decoated. Table 1 shows that ipil-ipil seed gum is still cream in color although the dark brown coat of the seeds was not removed. The very low % protein (<0.3%) (Table 2) is due to the denaturation of protein caused by boiling. Boiling also effectively removed mimosine. The % acid insoluble residue is higher (5.61%) but it is still within allowable range (not more than 7%). Acid insoluble matter may be due to the husk particles or seed coat that were not removed during the filtration of the aqueous solution. Crude fiber refers to acid- and alkali- insoluble residues. It has also a higher value equal to 9.05%.

**Table 1. Physical properties of purified L. leucocephala seed gum.**

| Physical Property | L. leucocephala Gum |
|---|---|
| Appearance (Form) | Powder |
| Color | cream - very light brown |
| Odor/Taste | slight beany odor |
| pH of 1% aqueous solution | 6.0 |

**Table 2. Chemical assay of L. leucocephala seed gum.**

| Component | % Assay | |
|---|---|---|
| | Ipil-Ipil Seed Gum (Purified by Fractional Precipitation) | Ipil-Ipil Seed Gum (Purified by Ion-Exchange Chrom,) |
| Acid Insoluble Residue | 5.61 | 4.0 |
| Protein | <0.3 | negative |
| Starch | negative | negative |
| Moisture | 8.55 | 10.0 |
| Crude Fiber | 9.05 | 5.8 |
| Total Ash | 2.1 | 1.3 |
| Gum Content | 74.39 | 78.9 |

% gum was calculated by subtracting from 100 the sum of the other components.

The Micronucleus Test is an *in vivo* method devised for screening chemicals for chromosome-breaking effects. Micronuclei are acentric chromatid and chromosome fragments that lag behind when the centric elements move towards the spindle poles (von Ledebur and Schmid, 1973).

The results of the Micronucleus Test (Table 3, Figure 1) showed that the ipil-ipil seed gums are not mutagenic at dosages of 3, 18, 35 and 50 mg / kg mouse. The number of MN-PCE in these four different dosages of ipil-ipil seed gum approximates that of water, the solvent control.

**Table 3. Mutagenicity and antimutagenic activity of the crude ipil-ipil seed gum using the micronucleus test.**

| Extract | Dose (mg/kg mouse) | Ave. No. of MN-PCE/ 1000 PCE (mean+/-SD) | % Inhibition |
|---|---|---|---|
| Tetracycline | 55.0 | 7.97 ± 2.75 | |
| Distilled water | | 2.77 ± 1.58 | |
| Ipil-Ipil gum | 2.94 | 3.47 ± 1.30 | |
| | 18.0 | 2.21 ± 1.53 | |
| | 35.0 | 3.64 ± 1.39 | |
| | 50.0 | 1.93 ± 0.96 | |
| Tetracycline + ipil-ipil gum | 2.94 | 2.60 ± 0.91 | 67.3% |
| | 18.0 | 3.70 ± 1.16 | 53.6% |
| | 35.0 | 2.25 ± 1.73 | 71.8% |
| | 50.0 | 1.13 ± 0.64 | 85.8% |

average of five mice per sample; three slides per mouse.

**Figure 1. Mutagenicity of ipil-ipil seed gum.**

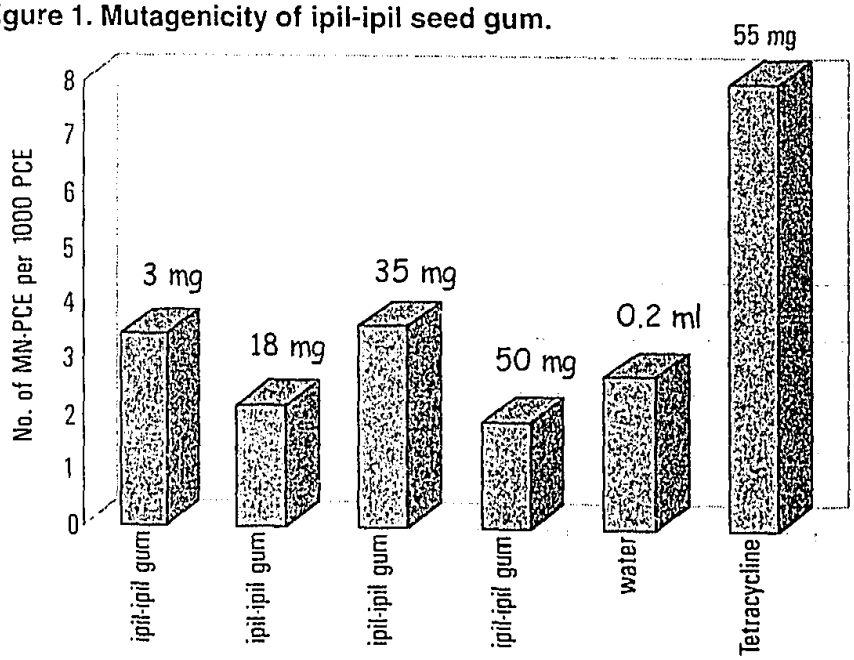
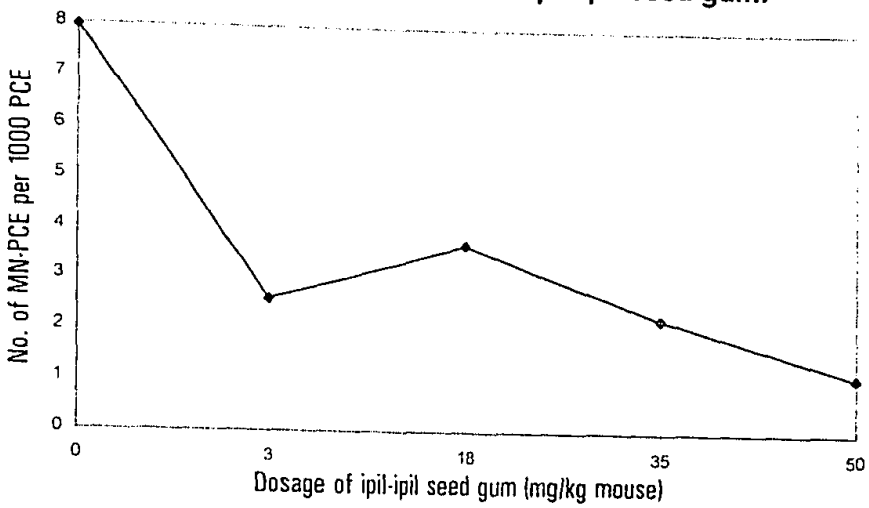


**Figure 2. Antimutagenicity potential of ipil-ipil seed gum.**




At a dosage of 3 mg / kg body weight, the pure isolate reduced by 67.3% the number of micronucleated polychromatic erythrocytes (MN-PCE) induced by the mutagen tetracycline (Table 3, Figure 2). An increase in the dosage of the gum to 50 mg / kg mouse led to an 85.8% reduction in MN-PCE. Thus the ipil-ipil seed gum is antimutagenic. Statistical analysis using Kruskal-Wallis One-Way Analysis of Variance by Ranks showed that the variances of (tetracycline + ipil-ipil seed gum) at dosages of 3, 35, and 50 mg / kg mouse are statistically different to that of the tetracycline, the positive control, at 0.001 significance level. At a dosage of 18 mg / kg mouse, the variance of (tetracycline + ipil-ipil seed gum) is statistically different to that of the tetracycline, the positive control, at $\alpha = 0.027$.

## CONCLUSION

The alternative procedure of isolating ipil-ipil seed gum by hot percolation of coated seeds is time- and cost-effective. Fractional precipitation can be used for purification. There is no need for ion-exchange chromatography as a decrease in % protein was already accomplished by boiling.

At dosages of 3, 18, 35 and 50 mg / kg mouse, the results of the Micronucleus Test showed that ipil-ipil seed gum is not mutagenic and is antimutagenic. This implies that ipil-ipil seed gum has no adverse effects on chromosomes and can even counteract the genotoxicity of chemical mutagens such as tetracycline.

## ACKNOWLEDGEMENT

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# Mutagenicity of Natural Pesticides on Bacterial Systems

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## ABSTRACT

*Different concentrations of alcohol-soluble crude extracts from leaves of two natural pesticides, marigold (Tagetes, erecta L.) and neem (Azadirachta indica A. Juss) were tested separately for genotoxicity using 3 auxotrophic tester strains of Salmonella typhimurium, TA 97 and TA 98 for frameshift mutations and TA 100 for base-pair substitution mutations. The extracts converted the mutant tester strains to the normal prototrophic states to form significant number of colonies on minimal glucose agar. Both extracts exhibited both frameshift and base-pair mutagenic activities.*

*Spontaneous mutations in very low frequency were observed in untreated plates of the three tester strains .pa.*

## INTRODUCTION

The drive for maximizing productivity and greater profit has given rise to the indiscriminate use of chemical pesticides. While this may have resulted in checking agricultural losses from harmful insects, studies have shown that some insects have grown resistant to these pesticides. As a consequence, more potent pesticides find their way to the market, with components that are increasingly more toxic to biological systems and with residues that pollute and are less friendly to the environment.

Research and other studies on natural/botanical pesticides are much needed these days considering the global need for environmentally safe pesticides. These would also contribute to the country's aim in making full use of or harnessing the full potential of its rich natural resources. The search for natural pesticides has also been triggered by the high cost of synthetic pesticides and the reported harmful effects of synthetic pesticides on non-target organisms like fish (Eisler, 1970) and man (Lim-Sylianco et. al., 1976).

Several plants have been used by local farmers as natural pesticides. Among the local species of pesticidal plants are the *Derris* (Maini and Morallo-Rejesus, 1993), *Tagetes* (Morallo-Rejesus et. al., 1993) and *Tinospora crispa* (Adalla et. al., 1993).

Among the *Tagetes* (marigold), the *Tagetes erecta L.* is the more commonly used species for its pesticidal properties. This is a strongly scented species whose flowers and/or leaves are pounded and mixed with water and used by farmers for their insecticidal properties. The flowers of the marigold contain a yellow crystalline substance reported by Quisumbing (1978) as quercetagetine $C_{27}H_{22}O_{13} + 4H_2O$.

A newly introduced pesticidal plant from India, the neem tree (*Azadirachta indica* A. Juss) is being extensively studied for the pesticidal activity of its seed (Maurer, 1983; Akou, 1987; Bham, 1983; Dryer, 1983; Jotunni et al., 1983; Saxena et al., 1983).

Recent researches on the chemical constituents of the neem seed extract (Kraus, 1991; Spharan et al., 1991) prove its efficacy for protection against insects. The main component in the methanolic extract of neem seeds is azadirachtin which was shown to be non-toxic among rats even at concentrations as high as 8500 mg/kg applied orally (Schmutterer and Zebitz, 1983).

To date, 2,000 plant species have been reported to possess pesticidal properties (Barroga-Jamias, 1989). However, only a few of these plants have been tapped in the manufacture of natural pesticides and for direct use by the farmers.

It is the objective of this study to explore the possible genotoxic effects of the extracts from the leaves of two natural pesticides, the marigold and the neem using the Modified Ames Test which makes use of specifically constructed mutant tester strains of *Salmonella typhimurium* as the test organisms.

## MATERIALS AND METHODS

Two specimens used as natural pesticides the *Tagetes erecta* L. commonly known as marigold (Eng.), *amarillo* (Spanish and Pilipino) and the *Azardirachta indica* A. Juss locally known as neem were used in this study. The identity of both species was verified by Mr. Leonard L. Co., of the Institute of Biology, University of the Philippines, Diliman, Quezon City. Specimens were deposited at the Herbarium of the Institute. The marigold plants were grown in the Botanical Garden and the neem leaves were taken from a tree in the garden, both at the Institute of Biology.

The Modified Ames Test (Maron and Ames, 1983) was used in this assay using the TA 97 and TA 98 for frameshift mutations and the TA 100 for base-pair substitution mutations. All three tester strains are histidine mutants of *Salmonella typhimurium* supplied by Dr. Bruce N. Ames of the University of California in Berkeley, U.S.A.

### Test for marigold

An alcohol-soluble extract from freshly harvested marigold leaves was prepared. The extract was prepared by using a 1:2 ratio (wt/vol) of plant material and 95% alcohol. The crude extract was centrifuged and the supernatant liquid was used for the experiment. Preliminary experiments were done based on a dose-response curve to determine the working concentrations which were $10^{-3}$, $10^{-5}$ and $10^{-7}$.

Twenty-four hours before the assay, three 125-ml flasks which contain 25 ml of nutrient broth were each innoculated with TA 97, TA 98 and TA 100, respectively. The flasks were then placed on a shaker at 20°C and were ready for the assay.

Bottom agar plates were prepared before the assay. This was done by dispensing 20 ml of minimal glucose agar (MGA) into sterile petri dishes and allowing the agar to harden.

To the melted top agar, 0.1 ml of the fresh culture of a specific strain and 1.0 ml of prepared crude extract were added, vortexed and poured immediately on the bottom plate containing 20 ml of MGA. The entire procedure was done in triplicate for each concentration for all the three tester strains.

Positive and negative control plates were prepared for each of the tester strains. For the positive controls, 2 plates were prepared: one MGA plate containing 0.5 ml mM as $NaN_3$ as the diagnostic mutagen and another MGA plate with histidine added. For the negative control, one MGA plate without histidine was prepared. All the MGA plates had the respective bacterial tester strain.

Scoring was done within 48 hours or as soon as visible colonies were seen.

Three trials were performed for each tester strain. For each trial, three replicates were made with triplicates for each replicate. The results were subjected to statistical analysis using Sandler's A test for significant levels.

The above experiment was performed separately for neem.

## RESULTS AND OBSERVATIONS

A summary of the three trials for the marigold extract is seen on Table 1. Trial I shows that the number of colonies formed decreased as the concentrations decreased from $10^{-3}$ to $10^{-7}$ in the three tester strains showing a dose-dependent relationship between the concentration of the crude marigold extract and the number of colonies formed. As the concentration of the extract was decreased, the number of the colonies formed decreased significantly in TA 97 in all the concentrations $10^{-3}$, $10^{-5}$

and $10^{-7}$ and in TA 100 the decrease was significant at $10^{-6}$ and $10^{-7}$ only. No significant decrease was observed in TA 98.

**Table 1. Average number of induced and spontaneous revertants in *Salmonella typhiumium* tester strains treated with extract from leaves of MARIGOLD (3 trials).**

| Tester strains | Trial | Average number of revertant colonies | | | | | |
|---|---|---|---|---|---|---|---|
| | | Positive control | | Negative control | Tester strains treated with MARIGOLD extract | | |
| | | $NaN_3$ | +His | -His | $10^{-5}$ | $10^{-6}$ | $10^{-7}$ |
| TA 97 | I | 192.3 | 328 | 22.1 | 297.3* | 201.3* | 205* |
| | II | 207 | 196 | 25.0 | 271.3* | 239* | 189* |
| | III | 198.2 | 193 | 75.0 | 279.3* | 238.4 | 221 |
| TA 98 | I | 216 | 601 | 5.0 | 288 | 199 | 130.3 |
| | II | 194.4 | 197 | 20.0 | 403* | 346.4* | 283.1* |
| | III | 258.2 | 270.3 | 79.0 | 421* | 368* | 308* |
| TA 100 | I | 252.6 | 459 | 49.0 | 495 | 357.4* | 359* |
| | II | 414 | 275.3 | 9.0 | 467.2* | 428.2* | 316.2* |
| | III | 225 | 293.4 | 90.0 | 477* | 452.1* | 404* |

*Significant at 5% level (Sandler's A Test)

The data of Trial II show that the number of colonies formed decreased as the concentration of the crude marigold extract decreased from $10^{-5}$ to $10^{-7}$. The decrease in the number of colonies was significant in all the three tester strains.

In Trial III, the data show that the number of colonies formed decreased as the concentration of the crude marigold extract decreased. Significant results were obtained in all the three concentrations in TA 98 and TA 100.

Table 2 shows the results obtained in neem. The data of Trial I show a significant increase in the number of colonies as the concentration of the extract decreased in all the three tester strains. This trend is also seen in Trial II and Trial III.

In all the three trials performed it is shown that as compared with the negative control, there is a significant increase in the number of colonies formed as the concentration of the neem extract is decreased. There is an inverse relationship between the concentration of the crude extract to the number of colonies formed. The higher the concentration of the crude extract the lesser the number or colonies formed.

Figures 1, 2 and 3 show negative (-) control plates with spontaneous revertant colonies. Induced revertant colonies are shown in TA 98 (Figure

**Table 2. Average number of induced and spontaneous revertants in *Salmonella typhiumium* tester strains treated with extract from leaves of NEEM (3 trials).**

| Tester strains | Trial | Average number of revertant colonies | | | | | |
|---|---|---|---|---|---|---|---|
| | | Positive control | | Negative control | Tester strains treated with MARIGOLD extract | | |
| | | $NaN_3$ | +His | -His | $10^{-3}$ | $10^{-5}$ | $10^{-7}$ |
| TA 97 | I | 190 | 214 | 8 | 267* | 404* | TNTC |
| | II | 566 | TNTC | 17 | 401* | 472* | TNTC |
| | III | TNTC | TNTC | 43 | 95* | 256* | 328* |
| | | | | | | | |
| TA 98 | I | TNTC | TNTC | 19 | 315* | 446* | TNTC |
| | II | TNTC | TNTC | 7 | 211* | 358* | TNTC |
| | III | TNTC | TNTC | 58 | 248* | 462* | TNTC |
| | | | | | | | |
| TA 100 | I | TNTC | 423 | 17 | 274* | 248* | 470* |
| | II | 196 | 281 | 59 | 269* | 331* | 354* |
| | III | TNTC | TNTC | 41 | 249* | 300* | 378* |

TNTC - Too numerous to count

*Significant at 5% level (Sandler's A Test)

4), TA 98 (Figure 5) and TA 100 (Figure 6) treated with different concentrations of crude marigold extract. Induced revertant colonies are also shown in Figures 7, 8 and 9 in TA 97, TA 9E3 and TA 100 respectively treated with different concentrations of crude neem extract.

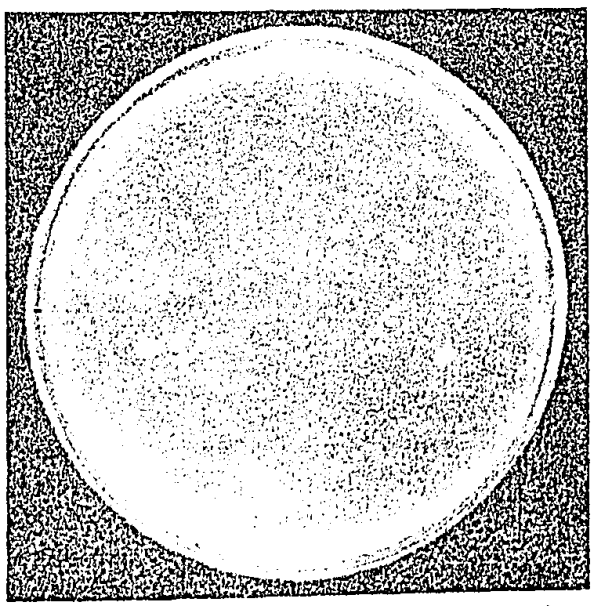

**Figure 1. Negative (-) control plate showing spontaneous revertant colonies in TA 97 *Salmonella typhimurium*.**

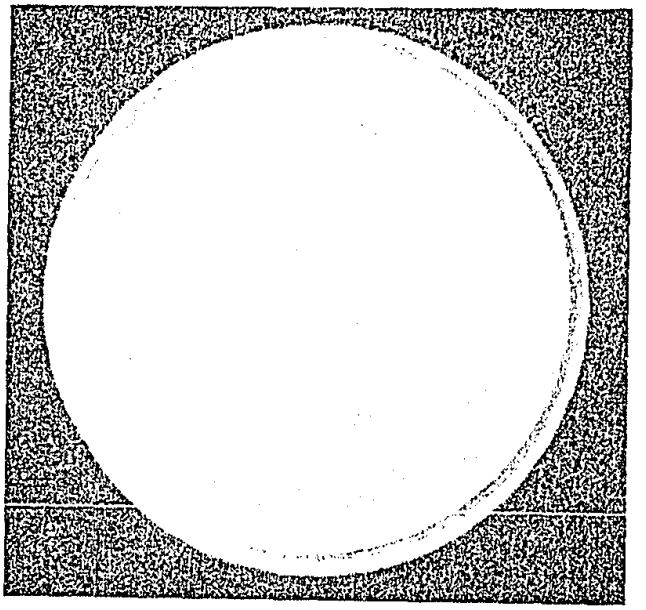

Figure 2. Negative (-) control plates showing spontaneous revertant colonies in TA 98 *Salmonella typhimurium.*

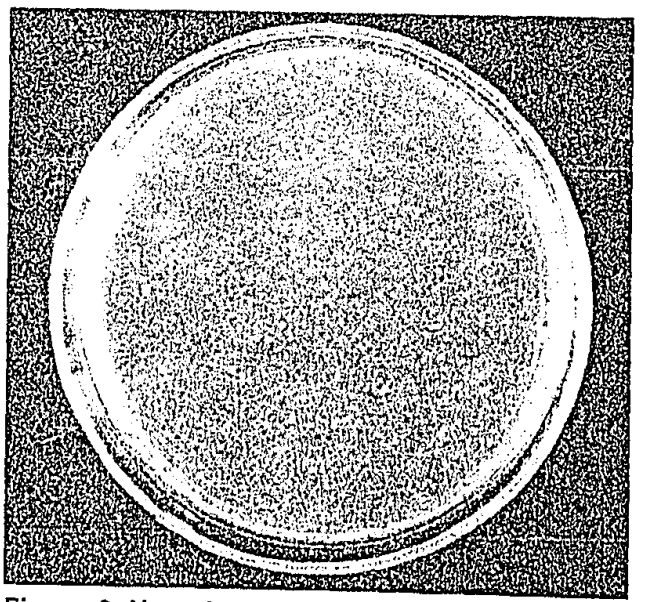

Figure 3. Negative (-) control plates showing spontaneous revertant colonies in TA 100 *Salmonella typhimurium.*

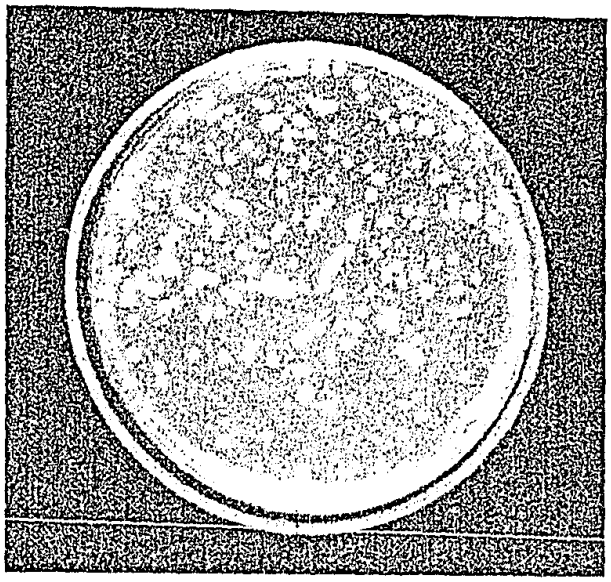

Figure 4. Revertant colonies in *Salmonella typhimurium* TA 97 treated with $10^{-7}$ crude extract of *Tagetes erecta* L. (marigold) leaves.

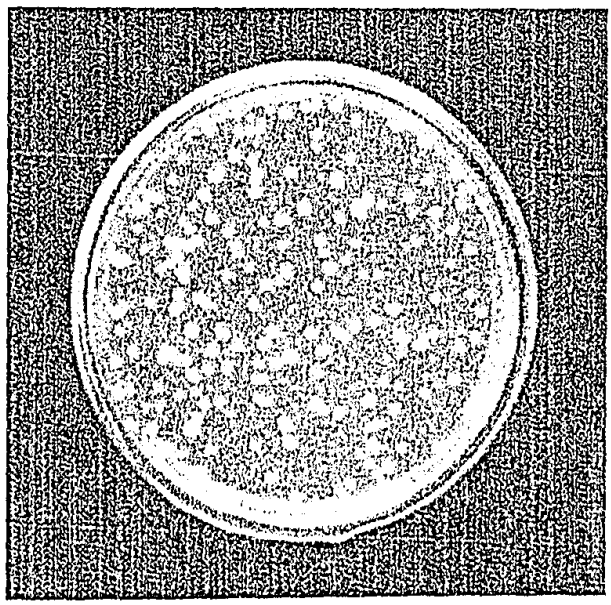

Figure 5. Revertant colonies in *Salmonella typhimurium* TA 98 treated with $10^{-3}$ crude extract of *Tagetes erecta* L. (marigold) leaves.

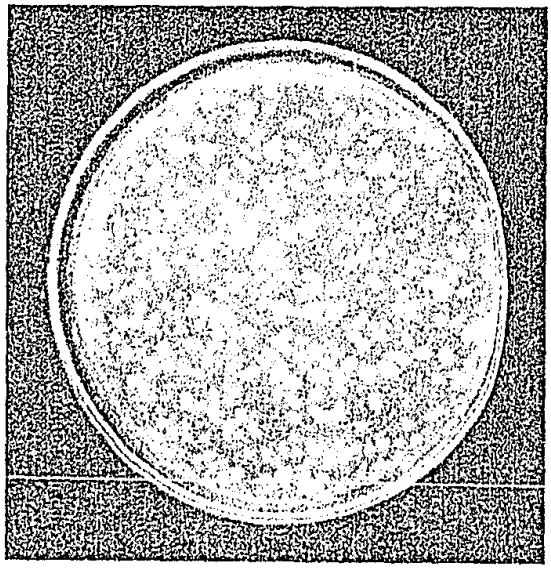

Figure 6. Revertant colonies in *Salmonella typhimurium* TA 100 treated with $10^{-3}$ crude extract of *Tagetes erecta* L. (marigold) leaves.

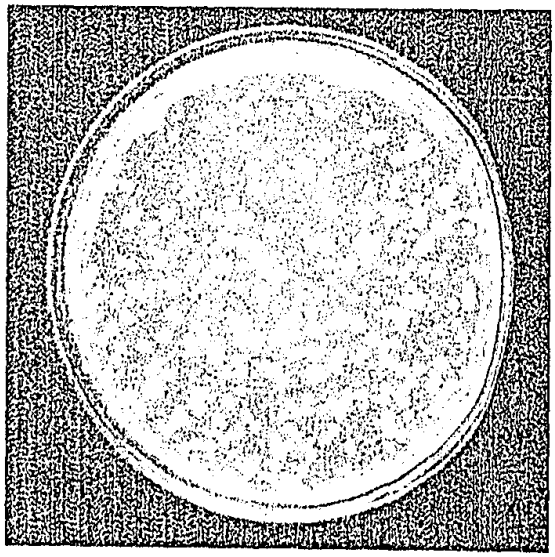

Figure 7. Revertant colonies in *Salmonella typhimurium* TA 97 treated with $10^{-3}$ crude extract of *Azadirachta indica* A. Juss (neem) leaves.

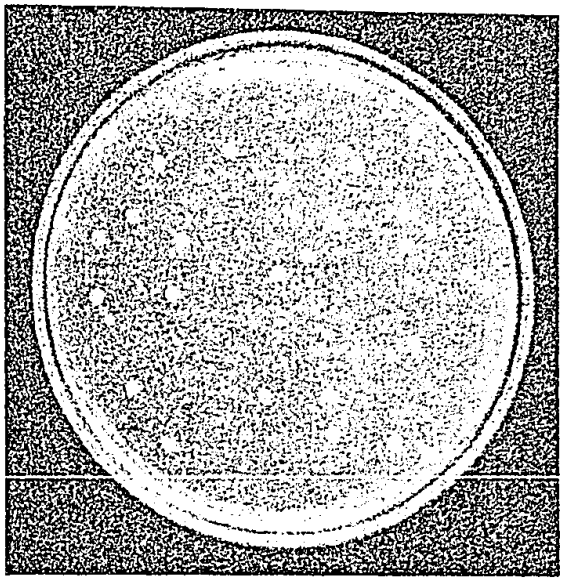

Figure 8. Revertant colonies in *Salmonella typhimurium* TA 98 treated with $10^{-3}$ crude extract of *Azadirachta indica* A. Juss (neem) leaves.

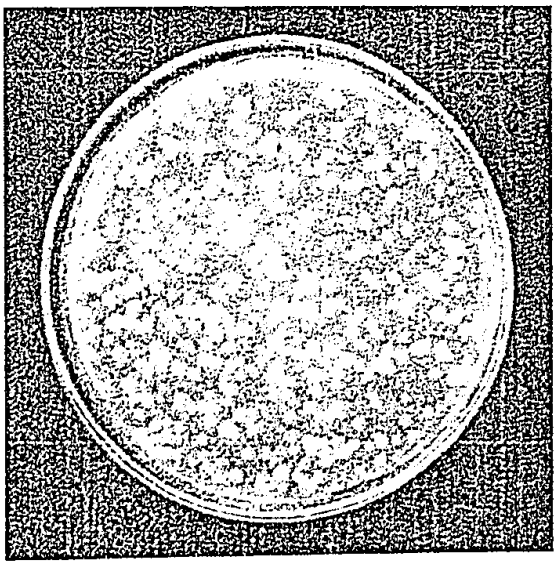

Figure 9. Revertant colonies in *Salmonella typhimurium* TA 100 treated with $10^{-3}$ crude extract of *Azadirachta indica* A. Juss (neem) leaves.

## DISCUSSION

From the foregoing results it has been shown that crude extracts from marigold and neem leaves proved to be mutagenic in the three tester strains of *Salmonella typhimurium* TA 97, TA 98 and TA 100. A component in the crude marigold and neem extracts was capable of producing frameshift and base-pair substitution type of mutations because it was able to produce revertant colonies (Figures 4, 5, 6, 7, E3 and 9) at the different concentrations used. It is possible that more than one component acting singly or syngergistically with each other brought about the results obtained in this work.

Auxotrophic frameshift tester strains can revert back to prototrophy by spontaneous and induced mutations. Induced mutations in this case are brought about by the presence of the crude extract from marigold and neem. A component from the crude extract brought reverse mutations such as transition (CG-TA) and transversion (CG-AT). These events make it possible for them to synthesize histidine and eventually from colonies in the extract-treated plates.

Another component present in the crude extracts of neem and marigold which reverted the mutant auxotrophic strains to the normal prototrophic state acted as a frameshift mutagen by intercalating in the DNA molecule inducing mispairing in the nucleotides. When such mispaired DNA replicates, there is probability of adding or deleting a base pair, thus generating a frameshift mutation.

The results obtained in marigold show that when the concentration of the crude extract of marigold is decreased the number of colonies decreased. A dose-dependent relationship is seen, as the concentration of the crude extract is decreased the number of colonies formed decreased. There is a direct relationship between the crude marigold extract and the number of colonies formed. The higher the concentration of the crude extract the higher the number of colonies formed (Table 1).

Patulin, a toxic antibiotic found in marigold was reported to be mutagenic by causing chromosomal damage in rats (Scott et al., 1972). Presumably it acts as a frameshift mutagen by intercalating in the DNA stack and inducing a mispairing in a string of nucleotides. When such mispaired DNA replicates, there is probability of adding or deleting a base pair in the nascent sequence thus generating a frameshift mutation.

Results obtained using the neem show that neem leaf extract exhibited mutagenic properties, however there is an inverse relationship between the concentration of the neem leaf extract and the number of colonies formed. As the concentration of the neem extract decreased there is an increase in the number of colonies formed (Table 2).

From the above results on the mutagenicity of neem leaf extract it can be concluded that a reliable prediction on the mutagenic effects of a crude alcohol extract from the neem leaves is not yet possible. The major chemical component of neem leaves is azadirachtin, which is a very efficient insecticide has been reported to be non-toxic to rats even at very high concentrations (Schmutterer and Zebitz, 1983). Work is needed to isolate and identify the mutagenic component of neem. It is beyond the scope of this present investigation. Antagonistic or synergistic effects of chemicals found in the crude extract are quite likely to be present in the neem leaf extract which gave an inverse relationship between the concentration and the number of colonies formed. The lower the concentration of the crude extract the higher the number of colonies formed.

Spontaneous revertant colonies were observed in the negative control of all the three tester strains. This type of reversion is naturally occurring and is brought about by spontaneous mutations that occur naturally in nature through tautomeric base mispairs and simple misaligment. Tautomeric base mispairs lead to base-pair mutations. These occur when the rare tautomeric forms of the bases mispair with other bases and results in transitions or transversions. Simple misalignment causes frameshift mutations.

## CONCLUSIONS

A component from each of the crude extracts of the marigold and the neem leaves induced the formation of revertant colonies in the three tester strains TA 97 and TA 98 both frameshift mutants and TA 100 a base-pair mutant indicating that the crude extract of the 2 species are both frameshift and base-pair mutants.

## ACKNOWLEDGEMENT

The author wishes to acknowledge the Natural Science Research Institute of the University of the Philippines in Diliman, Quezon City, (UP-NSRI) for the research grant to make this work possible.

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# On a New Species of the Genus Monorchis (Monticelli, 1893) Looss, 1902 (Family Monorchiidae) from India

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## ABSTRACT

*Nine trematodes of the genus Monorchis (Monticelli, 1893) Loos, 1902 were recovered from the intestine of Pomadasys hasta during collection of fish trematodes from Port Canning, India. These flukes are small, 0.81-1.00 mm long, 0.51-0.68 mm wide with spinose tegument larger oral sucker and 1:0.68 suckers ratio. Ovary pretesticular bean-shaped. Testis single on right side; vitellaria follicular with numerous bunches, each bunch with 12-14 follicles; excretory vesicle V-shaped, eggs 12-34 x 8-12 μ. The species is new to the genus and named as Monorchis bengalensis n.sp.*

## INTRODUCTION

During collection of trematodes of fishes from the Port Canning, South 24-Parganas, West Bengal, India nine specimens were recovered from the intestine of *Pomadasys hasta* (Pomadasyidae) which were identified as a new species to the genus *Monorchis* (Monticelli, 1893) Looss, 1902 under the family Monorchiidae Odhner, 1911. The genus *Monorchis* was established in the year 1893 by Monticelli and Looss (1902) described his type species *M. monorchis*. At present there are six valid species under this genus.

## MATERIALS AND METHODS

Fresh fish hosts were collected from the fishermen at Port Canning, West Bengal, immediately dissected out the intestine and viscera, kept in normal saline in separate petridishes to observe the trematode present. The trematodes after recovery were fixed in AFA under slight pressure of coverslip, preserved in 70% ethyl alcohol, stained with semichon's carmine, mounted in DPx, made camera lucida drawings and observed under microscope. All the measurements are based on five specimens and in mm unless otherwise mentioned. The data presented in the parentheses are mean with standard deviation of means.

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## RESULTS

Family : Monorchiidae Odhner, 1911
Subfamily : Monorchiinae (Odhner, 1911) Nicoll, 1915
Genus : *Monorchis* (Monticelli, 1893) Looss, 1902
Species : *Monorchis bengalensis* n.sp.
(Fig.1)

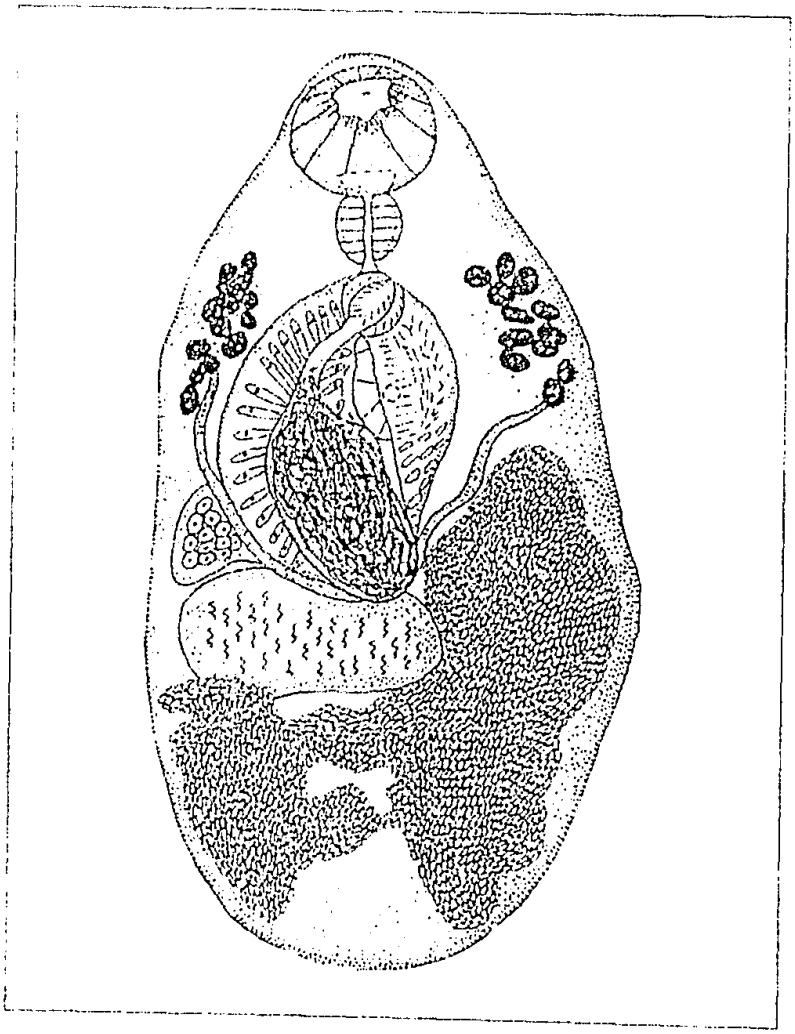

**Figure 1. Monorchis bengalensis n.sp. ventral view.**

Body small, 0.81-1.00 long (0.83±0.01), 0.51-0.68 wide (0.59±0.01). Tegument spinose. Ventral sucker 0.09-0.11 long (0.11±0.01), 0.08-0.11 wide (0.09±0.01), preequatorial, situated at 0.28-0.32 (0.30±0.02 from anterior end of the body. Oral sucker subterminal, 0.12-0.14 long (0.13±0.01), 0.14-0.15 wide (0.14±0.01), larger than ventral sucker. Sucker's ratio 1:0.68. Prepharynx small; pharynx 0.05-0.07 long

(0.06±0.01). 0.06-0.08 wide (0.07±0.01); oesophagus short; intestinal caeca extend upto posterior end of the body.

Testis single, 0.14-0.26 long (0.18±0.01), 0.18-0.30 wide (0.24±0.01), postacetabular, placed on right side of the body. Cirrus sac very large, pretesticular; seminal vesicle oval; pars prostatica long, surrounded by many prostate gland cells; cirrus globular, armed with spines. Genital pore near to pharynx; genital atrium without spines.

Ovary pretesticular, bean-shaped, 0.10-0.20 long (0.14±0.01), 0.14-0.17 wide (0.16±0.01), situated on right side of the body. Vitellaria follicular, oval, extracaecal in position forming bunches on both sides of the body. Each bunch consists of 12-14 follicles. Uterus mostly on hind portion of the body; terminal organ bipartite; anterior part thick walled, armed with spines. Uterus joins laterally. Eggs 12-34 x 8-12 µ.

Excretory vesicle 'V'-shaped.

**Host** *Pomadasys hasta* (Family Pomadasyidae)
**Location** Intestine
**Locality** Port Canning, South 24-Parganas, West Bengal, India
**Z.S.I. Reg. Nos.** W7908/1 to W7910/1
**Holotype** Deposited to the National Collection, Zoological Survey of India, Helminthology Section, Collection No. W7908/1.
**Paratype** Eight. Deposited to the National Collection, Zoological Survey of India, Helminthology Section Collection, No.W7909 & 10.

## DISCUSSION

Looss (1902) described *Monorchis monorchis* (Stossich, 1890) collected from *Cantharus orbicularis* and *Oblata melanura* at Triest, and also in *Blennius gattorugine, B. pavo, Diplodus sargus, Spicara alcedo, Chrysophrys lineata* and *C. aurata* at Mediterranean and *Smaris chryselis* from Black Sea and further he described *Monorchis parvus* from *Sargus annularis* and *S. rondeletti* at *Triest.*

Manter (1942) described *Monorchis latus* from *Anisotremus virginicus* and *Haemulon pulmieri* at Florida. Issa (1963) described *Monorchis hermani* recovered from *Chrysophrys aurata* from Egypt. Madhavi (1977) described *Monorchis minutus* from *Pomadasys maculatus* at Waltair Coast, Bay of Bengal. Bilquees (1981) described *Monorchis heterorchis* from Karachi Coast, Pakistan.

The comparison of the present species with other six valid species of the genus *Monorchis*, however, are made from the descriptions from literature, except *M. minutus* Madhavi, 1977, which is available (paratype) in the helminthological collection of Zoological Survey of India.

The present form collected from *Pomadasys hasta* from Matla estuary, Sunderbans, West Bengal, India is very much close to *M. minutus* Madhavi, 1977 but differs from it (Table 1) in the size of body; ratio of sucker, oral sucker being larger than ventral sucker; shape and position of ovary, ovary being bean-shaped; number of vitelline follicles, in each bunch 12-14; presence of 'V'-shaped excretory vesicle instead of 'Y'-shape. It is further differing from *M. monorchis* (Stossich, 1890), Looss, 1902, *M. parvus* Looss, 1902, *M. latus*, Manter, 1942, *M. hermani* Issa, 1963 and *M. heterorchis* Bilquees, 1981 in the ratio of sucker, shape of ovary, position of testis, number of vitelline follicles in each bunch, shape of excretory vesicle and size of eggs.

**Table 1. A comparison of M. bengalensis n.sp with a closer species of the genus Monorchis (Monticelli, 1893) Looss, 1902.**

| Characters | *M. minutus* Madhavi, 1977 | *M. bengalensis* n.sp. |
|---|---|---|
| Host | *Pomadasys maculatus* | *Pomadasys hasta* |
| Locality | Waltair Coast, Bay of Bengal, India | Port Canning, Matla estuary, Bay of Bengal, India |
| Body | | |
| length | 0.387 - 0.560 | 0.81 - 1.00 |
| width | 0.234 - 0.320 | 0.51 - 0.68 |
| Tegument | Spinose | Spinose |
| Oral sucker | | |
| length | 0.50 - 0.86 | 0.12 - 0.14 in diameter |
| width | 0.56 - 0.192 | |
| Prepharynx | Small or absent | Small |
| Pharynx | | |
| length | 0.003 - 0.039 | 0.05 - 0.07 |
| width | 0.043 - 0.051 | 0.06 - 0.08 |
| Ventral sucker | | |
| length | 0.059 - 0.092 diameter | 0.09 - 0.11 |
| width | | 0.08 - 0.11 |
| Caeca | Long | Extended to posterior end of the body |
| Testis | | |
| length | 0.082 - 0.105 | 0.14 - 0.26 in diameter |
| width | 0.076 - 0.117 | |
| Seminal vesicle | Oval | Oval |

**Continuation of Table 1.**

| Characters | *M. minutus* Madhavi, 1977 | *M. bengalensis* n.sp. |
|---|---|---|
| Ovary | | |
| length | 0.046 - 0.079 | 0.10 - 0.20 |
| width | 0.056 - 0.078 | 0.14 - 0.17 |
| Vitellaria | 8-11, follicles in two lateral bunches | 12 - 14, follicles in two lateral bunches |
| Eggs | | |
| length | 18 - 23 | 12 - 34 |
| width | 8 - 10 | 8 - 12 |
| Excretory vesicle | 'I'- Shaped | 'V'-shaped |

From the above discussion it is clear that the present form is new to science, and it is a first record from the Indian estuary. The authors suggest its name as *Monorchis bengalensis* as it has been collected from West Bengal.

## ACKNOWLEDGEMENT

The authors are grateful to the Head, Department of Zoology, University of Calcutta and the Director, Zoological Survey of India for their permission for continuation of the work and to the Deptt. of Science and Technology and NES, Govt. of West Bengal for financial assistance in the project.

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# Somatic Embryogenesis and Regeneration in Callus Derived from Gamma-Irradiated Scutellar Tissues of Two Varieties of Rice (Oryza sativa L.)

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## ABSTRACT

*High frequency of embryogenic calli induction and increased frequency of plantlet regeneration via somatic embryogenesis in two local indica rice cultivar SR26B and MW10 were evaluated from the gamma irradiated scutellar tissue. Out of two genotypes, SR26B exhibited better response than the genotype, MW10. Addition of casein hydrolysate though enhanced growth of the calli, yielded embryogenic calli as like as control. Intrinsic differentiation in the embryogenic calli has been demonstrated through the histological studies.*

## INTRODUCTION

The effect of gamma irradiation in mutation breeding of crop plants have been well established but little work has been done in the field of sensitivity of physical mutagen on callusing and regenerating properties. Evidences of low dose radiation on *in vitro* study induce stimulatory effect in a number of crop plants is on record (Arya and Hildebrandt, 1969; Saettler and Adams, 1970; Batra and Arya, 1974; Optrany, 1974; Degani and Pickholtz, 1980; Lin, 1982; Bajaj, 1970; Bajaj *et al*, 1970; Chaudhuri *et al*, 1995). This communication describes the results of investigation carried out on gamma irradiated scutellar tissue on *in vitro* culture with special reference to callus growth, somatic embryogenesis and regeneration potentiality.

## MATERIALS AND METHODS

Healthy dry seeds of indica rice SR26B (fine grained, tall and salt tolerant) and MW10 (dwarf, suitable for upland cultivation) were obtained from Rice Research Station, Chinsura, West Bengal and treated with increasing level of gamma radiation doses at 5 KR, 10KR, 15KR, 20KR from $^{60}$CO source.

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Scutellar tissues of embryos of dehusked seeds were used as explant, sterilised by 0.1% mercuric chloride followed by 70% ethyl alcohol and then placed in callus induction medium, i.e.MS (Murashige and Skoog's 1962) medium supplemented with 2,4-D(2mg/l).Callus was seperated from the scutellar portion and growth of the calli were measured in terms of 'Growth value' in different days intervals. Coconut water (CW) and casein hydrolysate (CH) were used as special additives. Cultures were observed for embryogenic callus formation with dissecting microscope. Calli showing embryogenic growth were placed in regenerating medium and photoperiod was maintained in diffuse light (8.7μ mol $m^{-2}s^{-1}$) for 14 hours during initiation and 16 hours at an intensity of 24μ mol $m^{-2}s^{-1}$ during shoot initiation.

Histological studies were carried out from the differentiating embryogenic tissue followed by the process of fixation in FAA [Formalin : Acetic acid : Alcohol (70%) at 1 : 1 : 10 v/v], passing in alcohol grades and finally embedding in paraffin. Sections were cut at 10-12 μm in a Spencer's rotatory microtome and were stained in toludene blue reagent.

## RESULTS

In both the cultivars calli induction were 100 percent in MS basal nutrient media supplemented with 2,4-D (2mg/l). Cultivar SR26B showed more profuse callus growth than MW10. Growth value of the callus was measured for both the cultivars in different days intervals i.e. 15,30,45 & 60 days respectively. Results showed that after 60 days intervals, the growth value increased at 5KR, 10KR and 15KR treatment in cultivar SR26B and slightly increased at 10KR treatment in cultivar MW10. (Table 1). Addition of

**TABLE 1. Effect of Gamma irradiation in Growth value of two cultivars in different days intervals.**

| Cultivar | Treatment | 15 days | 30 days | 45 days | 60 days |
|---|---|---|---|---|---|
| SR26B | Control | 5.45±0.85 | 7.94±1.50 | 12.5 ±1.42 | 26.0 ±3.49 |
| | 5KR | 5.78±1.01 | 8.39±1.19 | 17.64±1.40 | 31.59±2.48 |
| | 10KR | 5.56±0.9 | 8.87±1.24 | 17.82±2.41 | 34.08±2.46 |
| | 15KR | 5.30±0.75 | 7.68±1.04 | 15.4 ±2.26 | 30.68±5.19 |
| | 20KR | 5.26±1.16 | 7.62±2.56 | 12.46±1.48 | 26.82±3.52 |
| MW10 | Control | 2.55±0.42 | 3.84±0.82 | 6.50±1.25 | 11.9 ±2.64 |
| | 5KR | 2.62±0.42 | 3.99±0.54 | 7.39±1.82 | 12.28±1.18 |
| | 10KR | 2.78±0.33 | 4.40±0.66 | 7.75±0.75 | 14.55±1.16 |
| | 15KR | 2.75±0.34 | 4.38±0.74 | 7.09±0.24 | 12.54±0.94 |
| | 20KR | 2.65±0.40 | 4.06±1.22 | 7.41±1.58 | 12.20±1.42 |

Data represents mean ±SD of 10 replicate

$$\text{Growth value} = \frac{\text{Final Fresh wt - Initial Fresh wt.}}{\text{Initial wt.}}$$

casein hydrolysate (CH) results more profuse callusing along with rapid scutellum swelling.

After 30 days of culture, callus could be distinguished into two characteristic types. One of them was nodular having tightly packed cells and the other was friable containing loosely arranged cells similar to that of nonembryogenic calli as described by Nabors *et al* (1983).

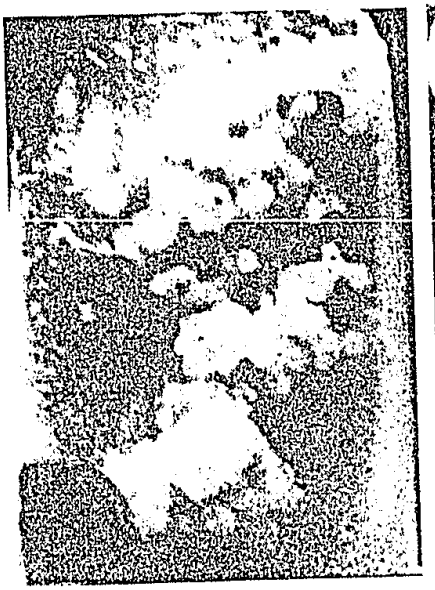

Figure 1. In vitro callus culture showing embryogenic calli.

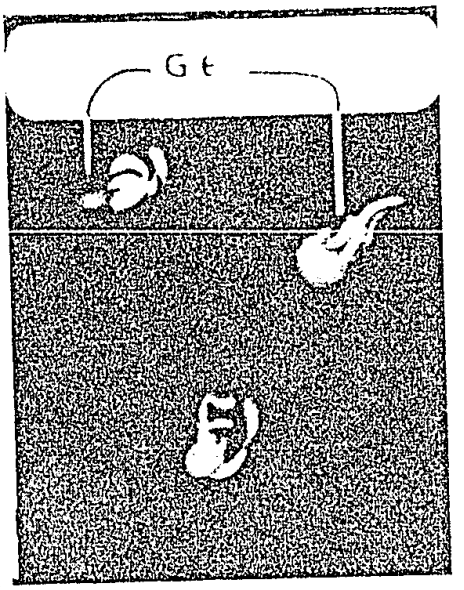


Figure 2. Germinating embryoids (GE).

Figure 3. Plantlet regeneration form the embryogenic calli.

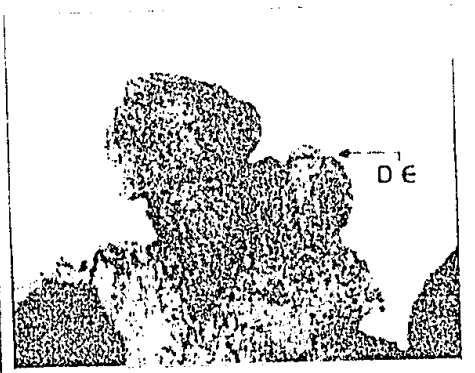


Figure 4. Section showing dome shaped embryoid with meristematic region.

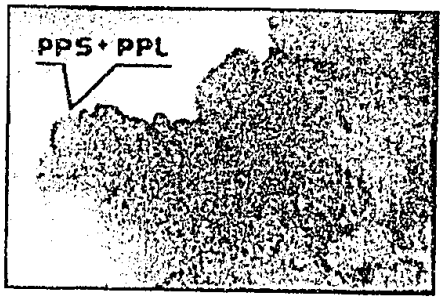


Figure 5. Section showing preprimordial shoot (PPS) and preprimoridal leaf (PPL).

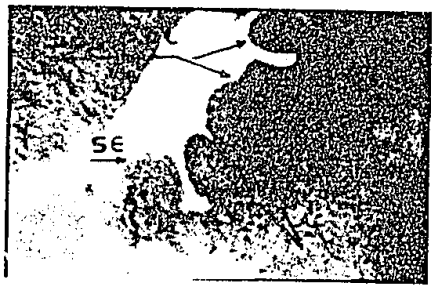


Figure 6. Section showing somatic embryoid (SE) and provascular meristematic region (PVM) with PPS and PPL.

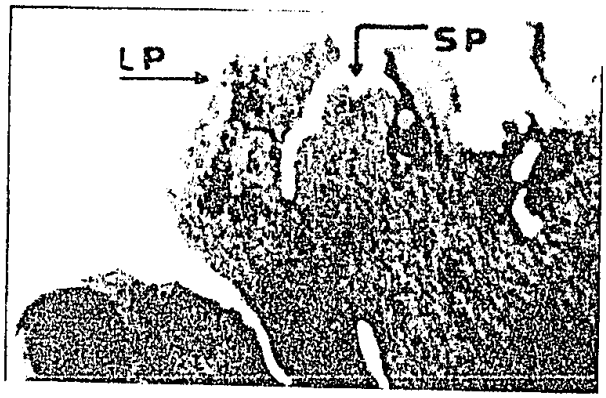


Figure 7. Section showing leaf primordia (LP) and shoot primordia (SP).

TABLE 2. Effect of Gamma irradiation on the productions of Somatic embryoids.

| Medium | Cultivars | Treatment | No. of immature Somatic embryoids | No. of germinating Somatic embryoids |
|---|---|---|---|---|
| MS+2,4-D(0.5 mg/l) + BAP 2mg/l) + CW (10% v/v)+ CH (500 mg/l) | SR26B | Control | 7.4±1.02 | 4±1.4 |
| | | 5KR | 7.8±1.24 | 6.1±1.2 |
| | | 10KR | 7.8±0.72 | 6.4±0.44 |
| | | 15KR | 7.4±0.84 | 5.6±0.24 |
| | | 20KR | 5.7±0.74 | 2.4±0.52 |
| | MW10 | Control | 6.2±1.32 | 3.2±1.26 |
| | | 5KR | 6.2±0.54 | 3.4±1.02 |
| | | 10KR | 6.4±0.22 | 3.6±1.18 |
| | | 15KR | 6.2±0.46 | 3.4±1.82 |
| | | 20KR | 3.4±0.74 | 1.8±0.22 |

Each value represents the mean ± SD of 10 replicable.
CW = Coconut water, CH = Casein hydrolysate,
Initial weight of tissue / culture tube = 100 mg ± 5 mg.

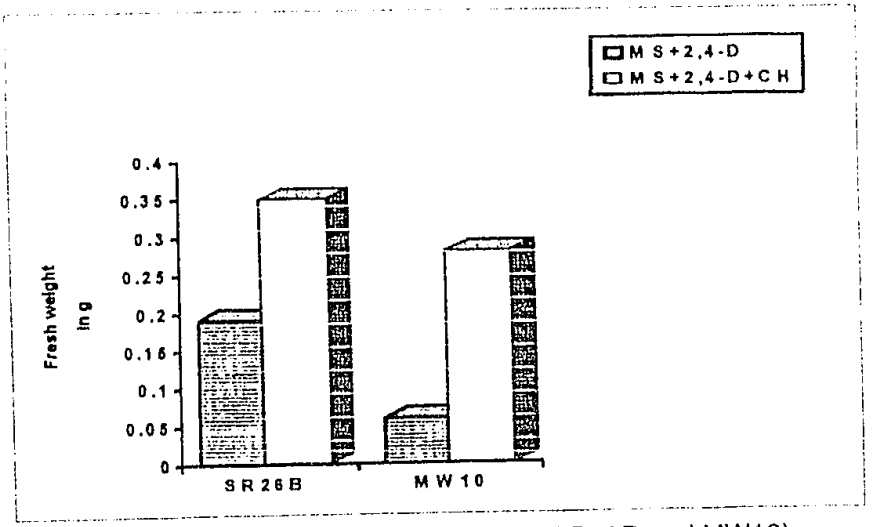


Figure 8. Callus growth of two rice cultivars (SR26B and MW10) on fresh weight basis for the different media constituents.

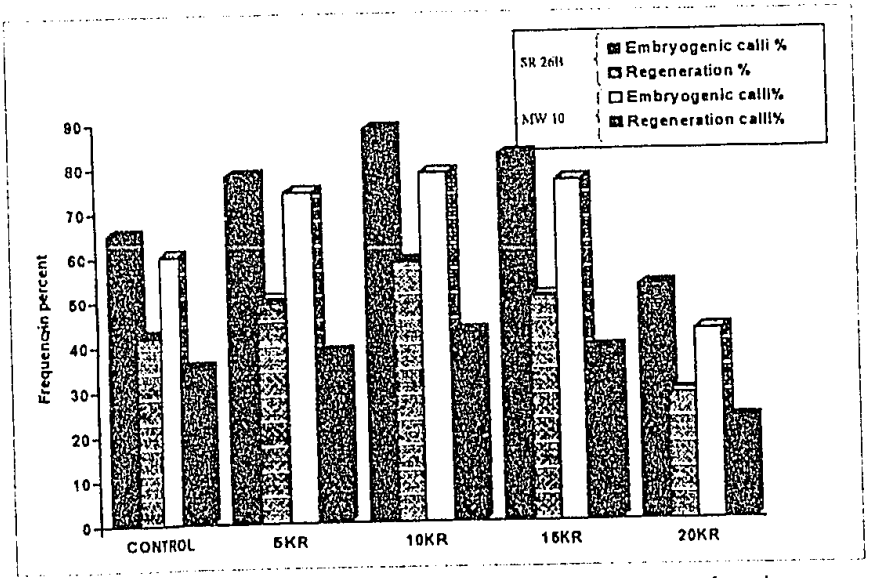


Figure 9. Effect of gamma-irradiation on the percentage of embryogenic calli formation and regeneration of two cultivars (SR26B and MW10).

Induction of treated explant (5KR, 10KR, 15KR, 20KR) also resulted an increase information of embryogenic calli specially at 5KR, 10 KR and 15Kr doses in MS basal nutrient media supplemented with 2,4-D (2mg/l). Fig.9 showed that treated explant in cultivar SR26B and MW10 exhibited development of more intensity of embryogenic calli at 5KR, 10KR and 15KR doses as compared to untreated sets. In between the two genotypes, cultivar SR26B showed better response than the cultivar MW10. It also appeared that casein hydrolysate promoted callus growth as represented by increased fresh weight but did not apparently support embryogenesis (Fig 8).

When the calli were transferred into regenerating medium [MS supplemented with 2,4-D(0.5mg/l)+BAP(2mg/l)+CW (10% v/v)+CH(500mg/l)] several green spots appeared and development of shoot and root occured soon after green spots were observed. Thus plantlets regeneration takes place via somatic embryogenesis by the germination of somatic embryoids. (Fig 1,2,3) It would be noted that albino plants were not observed in our culture. For the gamma-irradiated treated explant (Scutellar tissue) originated calli, when transferred into regenerating medium, number of immature somatic embryoids/culture tube in cultivar SR26B recorded 7.4±1.02 and number of germinating somatic embryoids/ culture tube counted 4±1.4. (Table 2). The best responses were observed at 10KR treatment where number of immature somatic embryoids recorded 7.8±0.72 and number of germinating somatic embryoids were 6.4±0.44.In cultivar MW10, number of immature somatic embryoids and number of germinating somatic embryoids were found to be 6.2±1.32 and 3.2±1.26 respectively. In this genotype also, best responses exhibited at 10KR treatment where number of immature somatic embryoids and germinating somatic embryoids were found to be 6.4±0.22 and 3.6±1.18. (Table 2).

Regeneration frequency was estimated 58% and 42% at 10Kr treatment as compared to the control (45% and 35%) in cultivar SR26B and MW10 respectively (Fig 9) (Table 3). Number of shoot developed /embryoid decreased at 15KR & 20KR treatment in cultivar SR26B. In cultivar MW10, number of shoot developed/embryoid decreased only at 20KR treatment (Table 3).

**TABLE 3. Morphogenic percentage from gamma-irradiated scutellar explant derived callus on regenerating medium.**

| Medium | Cultivars | Treatment | Morphogenesis (%)* | Number of Shoot **/ embryoids |
|---|---|---|---|---|
| MS+2,4-D(0.5 mg/l) + BAP (2mg/l)+ CW (10% v/v)+CH (500 mg/l) | SR26B | Control | 45 | 7.4±0.84 |
| | | 5KR | 50 | 7.4±0.26 |
| | | 10KR | 58 | 7.2±0.30 |
| | | 15KR | 50 | 5.8±0.42 |
| | | 20KR | 28 | 5.8±0.66 |
| | MW10 | Control | 35 | 5.4±1.44 |
| | | 5KR | 38 | 5.6±1.07 |
| | | 10KR | 42 | 5.6±0.46 |
| | | 15KR | 38 | 5.5±0.82 |
| | | 20KR | 24 | 4.2±0.62 |

* Data represents mean percentage of 10 replicate

** Data represents mean ±SD, initial wt of tissue/culture tube = 100 mg mg±5mg.

Histological observation of embryogenic calli in cultivar SR26B showed that the cells of the embryogenic calli were basophilic had thin walls and showed many meristematic regions of differentiated regions into proembryo like structures. Embryogenic calli initiated from scutellar tissue showed distinct meristematic areas in different zones. Globular meristematic centre or meristemoid were clearly visible in younger calli (Fig 4,5) which very often give rise to shoot primordia. Moreover, leaf primordia and incipient roots were found endogenously in calli. Bipolar structure were noticed which seemed like embryoids. Fig (4,5,6,7)

## DISCUSSION

Optimum concentration of 2,4-D (2 to 5mg/l) required for callus induction in indica cultivars has been well established (Raina *et al*, 1987; Kim *et al*, 1988; Raval and Chatto, 1993). In the present study, addition of casein hydrolysate showed an increase in callus growth. Similar report was suggested by Raval and Chatto, 1993. Growth value of the calli measured in different days intervals reflected that cultivar SR26B showed better response than the MW10. Calli originated from the gamma irradiated scutellar tissue resulted in an increased information of embryogenic calli percentage significantly in cultivar SR26B than the cultivar MW10. This phenomenon was comparable between the two cultivars and decreased in higher dose level. (Fig 9). Indica cultivars, indicate a significant diversity in growth response on MS media with or without supplements. The callus growth as well as regeneration was to be cultivar specific trait. Furthermore, percentage of calli showed somatic embryogenesis effected by gamma-irradiation provided a parameter which should be of value to involving a search for effective induction of mutagen. The increased number of immature somatic embryoids and germinating somatic embryoids particularly at 10KR treatment indicate that for both the cultivars, gamma-irradiation plays an important role in increasing the regeneration percentage through somatic embryogenesis. In higher doses i.e. at 20KR treatment this phenomenon was reversed. Many reports has already been suggested regarding the effect of radiation like X-rays, UV rays , g-rays on different organic compound and hormonal synthesis system in endogenous level. Szabo and Tajeda (1989) suggested the role of X-rays in the increasing production of ascorbic acid in horticultural plants (raddish, lettuce, tomato, capsicum, pea and bean). The role of UV rays in the increasing production of IAA in barley plants (Aknazarov and Shomanasurov, 1988) and *Spathyphyllum* (Rajagopal *et al*, 1987) has also been reported. Rajagopal *et al*, (1987) suggested that the interaction of ethylene, ABA, IAA and phenolic acids. The role of gamma irradiation in increasing phenolic production in castor bean has been reported by Reddy *et al* (1987). Stimulatory effect of callusing by gamma radiation also have been made in tobacco (Bar-or *et al* 1973) and Shamouti Orange (Spiegel and Kochba 1973) culture at 16KR treatment. Gamma radiation also plays

role in ethylene production in apple fruits (Zhao and Wan, 1989). Moreover, the role of IAA on increasing regeneration via somatic embryogenesis by using tryptophan (Precursor of IAA synthesis system) has already given by Peterson and Smith, 1991; Chaudhury *et al* 1993. It may be inferred from the review of literature, that in rice, gamma radiation particularly at 5KR, 10KR and 15KR dose level acts by altering the endogenous level of IAA or other accelerative substances which may cause the increase in embryogenic calli. However, it is necessary to monitor the alterations in endogenous levels of IAA synthesis for validating whether the phenomenon actually occurs during callus initiation or embryogenic calli formation and/or caulogenesis and rhizogenesis.

Differentiation in embryogenic calli has been demonstrated by the different stages like proembryo formation, shoot and leaf primordia with meristematic regions. Regeneration of whole plant through somatic embryogenesis in rice has been reported by Ling *et al*, 1983; Jones, 1985; Abe and Futsuhara, 1986; Wang *et al.*, 1987; Sticklen, 1991; Rueb *et al.*, 1994. Increase of regeneration via somatic embryogenesis seeks further investigation for the assessment of particular reasons behind it.

## ACKNOWLEDGEMENTS

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# High Temperature Stress on Certain Aspects of Protein Metabolism in the Hemolymph, Fat Body, Silkgland and on Larval Developmental Period, Body Weight and Economic Characters of Silkworm, *Bombyx mori L.*

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## ABSTRACT

*Effect of high temperature stress on protein metabolism of hemolymph, fat body, silkgland and on larval body weight and economic characters of silkworm, Bombyx mori, were studied after continuous exposure of post IV moult larvae to high temperature (33±1°C) for a period of 24, 72, 120, and 168 hours with the controls kept at 24±1°C.*

*Heat stress on silkworm hemolymph, fat body and silkgland caused significant reduction in total protein and free amino acid contents followed by a concomitant increase in protease activity. A steady enhancement in the activities of alanine aminotransferase (AlAT) and aspartate aminotransferase (AAT) paralleled the elevation of glutamate dehydrogenase (GDH) activity in the tissues studied. All these changes clearly documented the induction of severe proteolysis.*

*The larval developmental period (larval duration), larval body weight, single cocoon weight, single shell weight, fecundity and average filament length showed significant reductions when the larvae were exposed to high temperature.*

## INTRODUCTION

Environmental temperature affects all levels of the biology of an organism, from fundamental biochemical processes at the sub-cellular level to evolutionary consequences of population dynamics (Prosser, 1986). Thus, temperature determines life of organism more than many other environmental factors. Longer periods below or above a critical temperature causes irreversible destruction of bioactive structure and function and lead to a disorganization of metabolic processes. (Hoffman, 1985).

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The thermal history of an insect can have profound effects upon subsequent development stages of that insect. At extremely high temperature, death is immediate, but at lower temperature, death may occur much later or the insect may in some other manner be affected. For example, high temperature exposure during the prepupal stage and pupal stage of silkworm. *Bombyx mori* reduces growth, development, fecundity or causes complete sterility (Sugai and Kiguchi, 1967, Sugai and Takahashi, 1981, Pillai and Krishnaswami, 1987).

India, being tropical country, most of the places where the silkworm rearings are conducted for commercial production of raw silk as well as seed production show a great degree of variations in temperature (Pillai and Krishnaswami, 1987, Benchamin and Reddy, 1989a, b). The temperature acts not only the role of all chemical reactions, but also causes conformational transition of proteins, enzymes etc. (Somme, 1972, Alexandrov, 1977, Reddy and Benchamin, 1992). Earlier workers had established that increase in temperature entails a greater demand for energy to meet the metabolic requirements (Hochachka and Somero, 1971). Although no studies have been reported so far on the metabolism of various tissues of silkworm *Bombyx mori* with reference to major sericultural zones like Andhra Pradesh and Tamil Nadu States of India, where extreme high temperature (32°C to 46°C out side environment temperature; 31°C to 35°C room temperature) prevails during the months of late February to July. Therefore, the present study was aimed at elucidating the possible alterations in the protein metabolism of functionally important tissues, larval weight and economic characters in heat stressed 5th instar growing silkworms.

## MATERIALS AND METHODS

### Insect rearing

Disease free layings of $NB_4D_2$ race were procured from Andhra Pradesh State Government Grainage and used in the present study.

Freshly hatched larvae of *Bombyx mori* were reared till up to IV moult on the leaves of *Morus alba* ($M_5$ variety) following standard rearing conditions (Krishnaswami, 1978).

### Experimental procedure

Immediately after IV moult, the test individuals of same size and age were collected from the rearing tray and divided into six batches of 100 larvae each. These were transferred to the environmental chamber at 33±1°C and 58±1% R.H., the prevailing room temperature and humidity respectively during the summer season. The larvae were fed *ad libitum* four times a day up to their spinning on mulberry leaves. Another set of six batches were maintained separately at standard rearing conditions of 24±1°C and 75±5% relative humidity (Krishnaswami, 1978). These served as control

batches. Six worms each from control and experimental batches were sacrificed on day 1 (after 24 hrs of exposure), day 3 (after 72 hrs of exposure), day 5 (after 120 hrs of exposure) and day 7 (after 168 hrs of exposure) for the biochemical analysis.

### Hemolymph, fat body, and silkgland collection

Hemolymph was collected on day 1, day 3, day 5, and day 7 separately from the control and experimental larvae by cutting first pair of prolegs. The hemolymph from the six worms of each batch were pooled and collected in pre-chilled glass vials containing few crystals of phenylthiourea as an anti-coagulant. The hemolymph thus collected was used immediately for biochemical analysis.

After collection of hemolymph, the fat body and silkgland from both control and experimental silkworms were dissected out in ice-cold Bombyx saline (Yamaoka et al., 1971) after making longitudinal mid-ventral incision along the entire body length. The posterior silkgland and freely floating fat bodies from the abdominal region of the insect was carefully excised and then blotted. After the tissues were weighed, these were used immediately for the biochemical assays.

### Biochemical assays of protein metabolism

The total protein content in the hemolymph, fat body and silkgland were estimated by the method of Lowry et al., (1951) using bovine serum albumen as standard.

Free amino acids were estimated in the tissues by the method of Moore and Stein (1954).

Protease activity was measured by the method of Davis and Smith (1955). The reaction mixture contained 0.5 ml 1% casein, 2 ml of 0.1 M phosphate buffer (pH 7.0) and 2 ml of enzyme source prepared from 2% tissue homogenates in cold distilled water. The contents were mixed and incubated at 30°C for 30 min. The reaction was stopped by adding 2 ml of 2% ninhydrin reagent. For the estimation in hemolymph, 0.2 ml of hemolymph is added to the reaction mixture and run similarily. The protease activity is expressed as micromoles amino acid nitrogen released/ mg protein/h.

Activities of alanine and aspartate aminotransferases were assayed by the method of Reitman and Frankel (1957). The reaction mixture for AlAT contained 100 micromoles of phosphate buffer (pH 7.4), 2 micromoles of α- ketoglutarate, 100 micromoles of alanine and 0.2 ml enyzme source prepared from 1% tissue homogenates in 0.25 M sucrose. For AAT the reaction mixture was same as that of AlAT except for alanine, aspartic acid was used. The reaction mixture was incubated at 37°C for 30 min. The reaction was stopped by the addition of 1 ml of 0.001 M 2, 4-

dinotrophenylhydrazine (ketone reagent). For the estimation in hemolymph, 0.2 ml of hemolymph is added to the reaction mixture and run similarily. The alanine aminotransferase activity is expressed as micromoles pyruvate formed/mg protein/h. and the aspartate aminotransferase activity as micromole oxaloacetate formed/mg protein/h.

Glutamate dehydrogenase activity was estimated using the method of Lee and Lardy (1965). The reaction mixture contained 1 ml of 0.4 M phosphate buffer (pH 7.4), 0.5 ml of 0.1 M sodium glutamate, 0.1 ml 0.0001 M NAD, 1 ml 0.004 M INT and 0.5 ml dialyzed extract prepared from 2% homogenates in 0.25 M sucrose solution. The reaction mixture was incubated at 37°C for 30 min and the reaction was stopped by adding 6 ml of glacial acetic acid. For the estimation in hemolymph, 0.2 ml of hemolymph is added to the reaction mixture and run similarly. The GDH activity was expressed as micromoles formazon formed/mg protein/h.

## Determination of larval duration, larval body weight and economic characters

**Larval duration** - The 5th instar larval duration was calculated from the beginning of the 5th instar to larval spinning and expressed in hours (h).

**Larval body weight** - The larval body weight was determined by taking weights of ten larvae just prior to spinning stage on electronic balance and expressed as g wet weight/10 mature larvae.

**Single cocoon weight** - Cocoons of 10 females and 10 males were weighed singly or individually on electronic balance, then average weight expressed in grams (g) was calculated.

**Single shell weight** - Shells of 10 females and 10 males (used for single cocoon weight determination) were weighed individually or single on electronic balance and then, average weight expressed in grams (g).

**Fecundity** - Female moths emerging from the pupae of control and experimental batches were paired with the males of corresponding age obtained from control batches. The eggs laid female moths were counted and expressed in number.

**Average filament length** - Silk is extracted from the outer layer of cocoon in the form of a long filament through reeling process with the help of multi-end relling machine. The length was measured in meters (m).

## Statistical analysis

The mean of six individual values were subjected to statistical treatments. Student's t-test was used to compare the differences between the control and experimental groups. The significance level was derived at $P<0.05$ in all cases.

## RESULTS

The results obtained in the present study are documented in the Tables 1 and 2, and Figures 1 to 4. With exposure to high temperature, the total protein and free amino acid contents in the hemolymph, fat body and

**Table 1. Total proteins and free amino acids in the hemolymph (mg/100 ml), fat body (mg/g wet weight) and silkgland (mg/g wet weight) in control (24±1°C) and heat stressed (33±1°C) silkworks.**

| | 24 hrs | | 72 hrs | | 120 hrs | | 168 hrs | |
|---|---|---|---|---|---|---|---|---|
| Tissue | Cont. | Expt. | Cont. | Expt. | Cont. | Expt. | Cont. | Expt. |
| | | | | TOTAL PROTEINS | | | | |
| Hemolymph | 4587 ±198 | 4168 ±125 | 5471 ±289 | 4114 ±181 | 6003 ±304 | 3728 ±204 | 6452 ±325 | 3157 ±197 |
| | (-9.13%)b | | (-24.80%)b | | (-37.89%)d | | (-51.72%)d | |
| Fat body | 164.42 ±13.62 | 141.15 ±10.12 | 192.40 ±12.91 | 131.37 ±8.93 | 221.89 ±19.30 | 115.80 ±11.81 | 238.80 ±20.95 | 84.18 ±6.92 |
| | (-14.15%)a | | (-31.72%)d | | (-47.81%)d | | (-64.75%)d | |
| Silkgland | 197.69 ±14.21 | 181.49 ±13.01 | 231.45 ±21.92 | 183.23 ±12.72 | 275.61 ±27.87 | 184.33 ±11.33 | 306.82 ±31.84 | 162.95 ±14.92 |
| | (-8.19%)NS | | (-20.83%)NS | | (-20.83%)c | | (-46.89%)d | |
| | | | | FREE AMINO ACIDS | | | | |
| Hemolymph | 524.80 ±41.75 | 468.59 ±38.95 | 598.41 ±41.75 | 464.31 ±36.87 | 674.74 ±61.32 | 439.99 ±34.98 | 728.80 ±65.41 | 385.17 ±33.98 |
| | (-10.71%)NS | | (-22.41%)c | | (-34.79%)d | | (-47.15%)d | |
| Fat body | 32.21 ±2.62 | 28.09 ±1.22 | 38.71 ±3.02 | 27.13 ±2.89 | 47.13 ±5.12 | 25.82 ±2.12 | 53.31 ±4.82 | 19.76 ±1.04 |
| | (-12.79%)a | | (-29.91%)c | | (-45.21%)d | | (-62.93%)d | |
| Silkgland | 21.45 ±1.71 | 19.34 ±0.89 | 27.91 ±2.17 | 22.62 ±1.89 | 31.87 ±2.94 | 21.71 ±1.58 | 36.57 ±3.71 | 20.54 ±1.01 |
| | (-9.84%)NS | | (-18.95%)c | | (-31.88%)c | | (-43.83%)d | |

Percentage Decrease relative to controls is given in parentheses.
Each value is ± mean SD (n=6).
a P<0.05; b P<0.02; c P<0.01; d P<0.001; NS - not significant.

silkgland of silkworms were significantly decreased at 24th, 72nd , 120th and 168th hours (Table 1). Protease activity was significantly increased in the tissues of the silkworms at all exposure periods (Figure 1). The activities of alanine and aspartate aminotransferases (AlAT and AAT) were also elevated significantly in the tissues under study (Figure 2 and 3). Correspondingly, the glutamate dehydrogenase (GDH) were also increased (Figure 4). Based on the values obtained from the present inquiry, the

**Table 2. Larval duration, larval body weight, single cocoon weight, single shell weight, average filament length and fecundity in control (24±1°C) and heat stressed (33±1°C) silkworms.**

| Larval duration (h) | | Larval body weight (g wet wt/10 mature larvae) | | Single cocoon weight (g) | | Single sheel weight (g) | | Average *filament* length (m) | | *Fecundity (By number)* | |
|---|---|---|---|---|---|---|---|---|---|---|---|
| Cont. | Expt. | Cont. | Expt. | Cont. | Expt. | Cont. | Expt. | Cont. | Expt. | Cont. | Expt. |
| 168 | 156 | 48.415 | 28.947 | 1.824 | 1.061 | 0.362 | 0.228 | 840 | 562 | 467 | 271 |
| ±006 | ±003 | ±2.758 | ±1.402 | ±0.189 | ±0.074 | ±0.039 | ±0.023 | ±64 | ±49 | ±41 | ±21 |
| (-7.14%)b | | (-40.21%)d | | (-41.83%)d | | (-37.02%)c | | (-33.0%)d | | (-42.0%)d | |

Percentage Decrease relative to controls is given in parentheses.
Each value is ± mean SD (n=6).
b P<0.02; c P<0.01; d P<0.001.

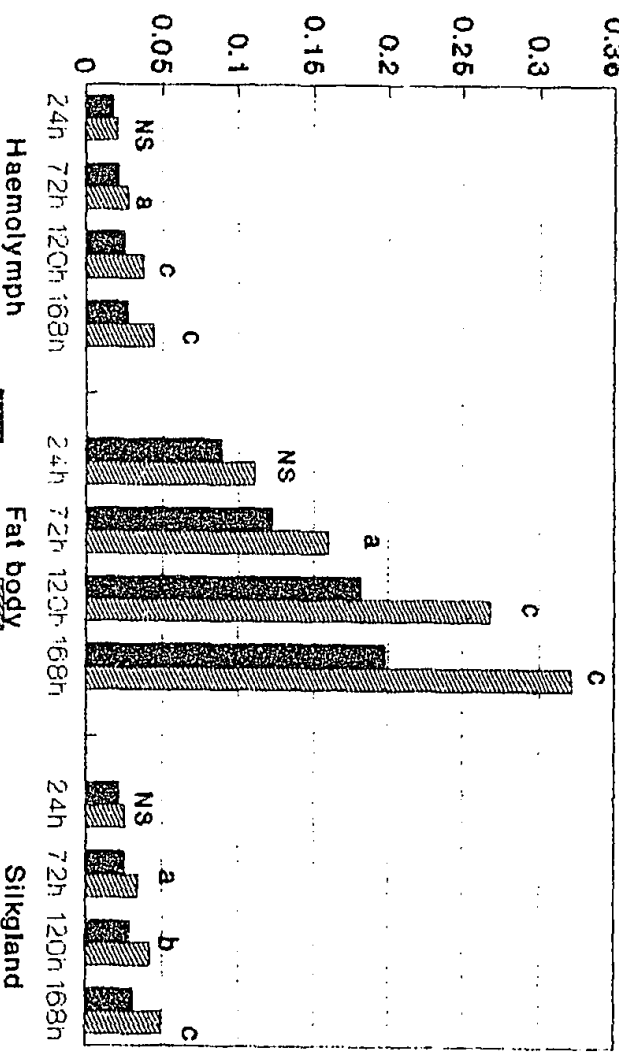


Figure 1. Effect of high temperature stress on protease activity in the hemolymph, fat body and silkgland at 24 hrs, 72 hrs, 120 hrs and 168 hrs of post IV moult larvae of control (24±1°C) and experimental (33±1°C) silkworms. Signs on each bar indicates, a- Significant at P<0.05; b- Significant at P>0.02; c- Significant at P>0.01; NS-Not significant.

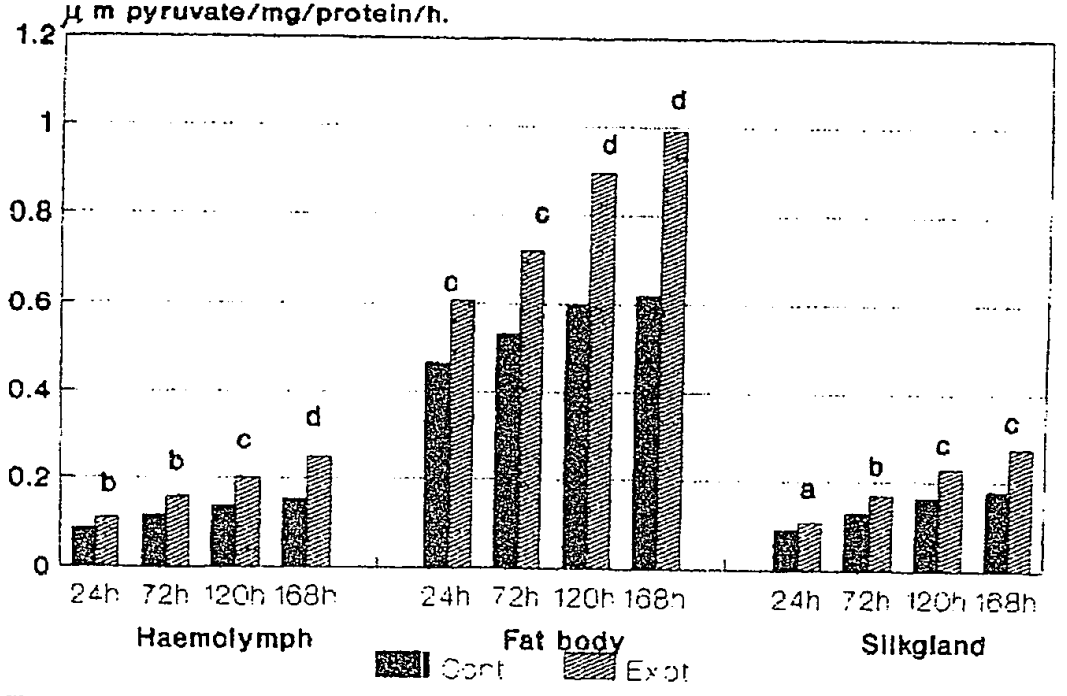


Figure 2. Effect of high temperature stress on alanine aminotransferase (AlAT) activity in the hemolymph, fat body and silkgland at 24 hrs, 72 hrs, 120 hrs and 168 hrs of post IV moult larvae of control (24±1°C) and experimental (33±1°C) silkworms. Signs on each bar indicates, a-Significant at P<0.05; b-Significant at P<0.02; c-Significant at P<0.01; d-Significant at P<0.001; NS-Not Significant.

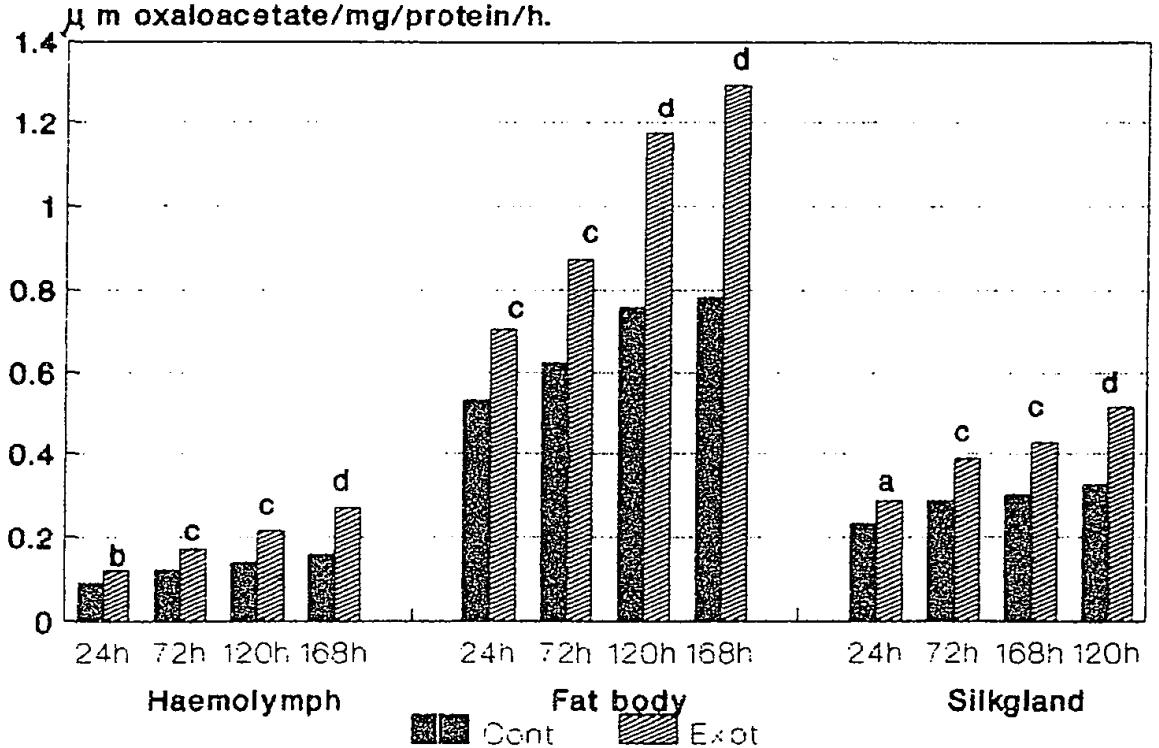


Figure 3. Effect of high temperature stress on aspartate aminotransferase (AlAT) activity in the hemolymph, fat body and silkgland at 24 hrs, 72 hrs, 120 hrs and 168 hrs of post IV moult larvae of control (24±1°C) and experimental (33±1°C) silkworms. Signs on each bar indicates, a - Significant at P<0.05; b - Significant at P<0.02; c - Significant at P<0.01; d - Significant at P<0.001; NS - Not Significant.

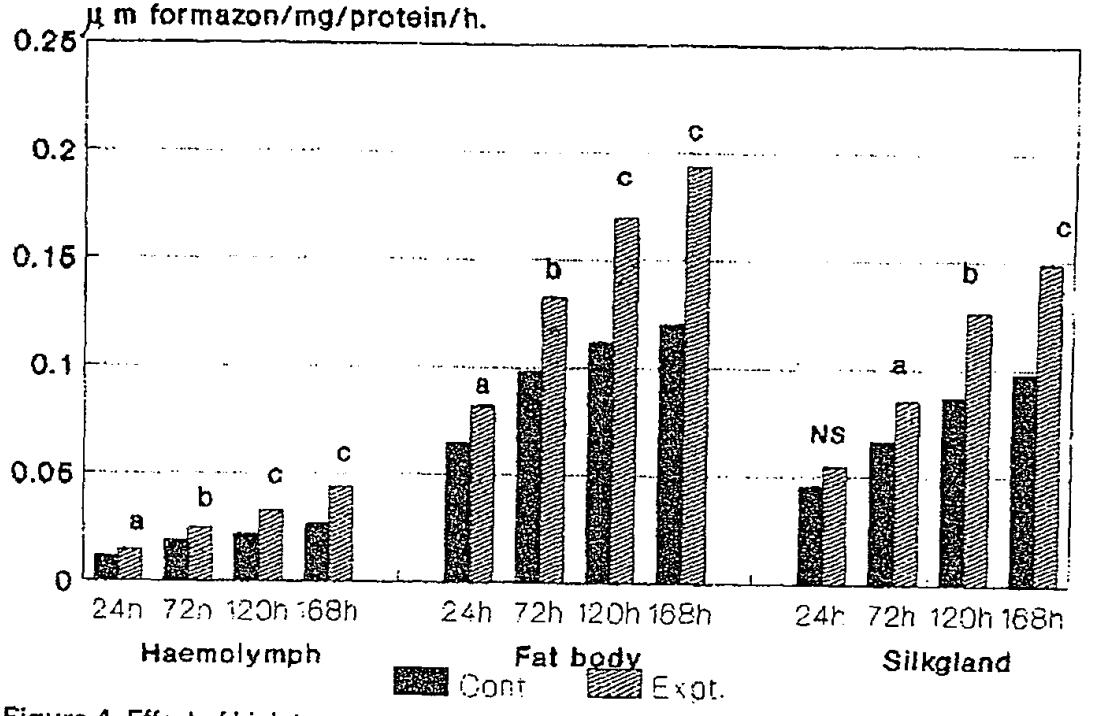


Figure 4. Effect of high temperature stress on glutamate dehydrogenase (GDH) activity in the hemolymph, fat body and silkgland at 24 hrs, 72 hrs, 120 hrs and 168 hrs of post IV moult larvae of control (24±1°C) and experimental (33±1°C) silkworms. Signs on each bar indicates, a - Significant at P<0.05; b - Significant at P<0.02; c - Significant at P<0.01; d - Significant at P<0.001; NS - Not Significant.

magnitude in the changes in all the parameters studied increases with time. Total 5th instar larval duration, larval body weight, single cocoon weight, single shell weight, fecundity, and average filament length of silkworm *Bombyx mori* showed marked reduction under high temperature stress (Table 2).

## DISCUSSION

Proteins being the key organic constituents, their role in the compensatory mechanisms of silkworm is vital, specially during stress. Environmental stress could evoke compensatory metabolic changes through modification and modulation of the quantity of proteins. Studies on the impact of temperature, particularly high temperature on metabolism of silkworms are very few (Reddy and Benchamin, 1990, 1992). In the present study a progressive depletion in total proteins in the hemolymph, fat body and silkgland is observed in silkworms subjected to high temperature, suggesting the existence of a high protein hydrolytic activity, which could be due to impairment of the protein synthesis. The decrease in protein levels could also be due to their breakdown/degradation. The degradation products may inturn be fed into tricarboxylic acid cycle through the aminotransferase system to cope up with the high energy demands augmented during high temperature stress. Maintenance of proteins in a highly organized state requires an active and continuous supply of energy. If this is impaired, the organ structures break down and the proteins partially get denatured in their configuration. Further, depletion in free amino acid pool in the hemolymph, fat body and silkgland, indicates the mobilization of free amino acids into oxidative metabolism to overcome stress conditions. These findings are in agreement with the earlier report wherein the authors reported significant depletion in total protein and free amino acid levels in the testes of silkworms under high temperature stress conditions (Reddy and Benchamin, 1992).

The decrease in total protein levels, along with an increase in protease activity in the hemolymph, fat body and silkgland, may indicate an intensive proteolytic activity in the tissues of the silkworms exposed to high temperature. Increase in protease activity could be due to massive lysis of tissues or may be due to the damage caused to lysosomes.

The alanine and aspartate aminotransferases serve as a strategic link between the carbohydrate and protein metabolism under environmental stress (Marting et al., 1981). The observed elevation in the activities of AlAT and AAT in the hemolymph, fat body and silkgland in this study offers an excellent support to the suggestion that decrease in free amino acid pool is due to their mobilization into transamination activities. Since the activity of AlAT forms a general index of amino oxidations and AAT activity points to mobilization of amino acids into gluconeogenesis (Meister, 1965, Mane and Mehrota, 1977, Reddy and Benchamin, 1992),

the observed elevation in the activities of these enzymes suggest the mobilization of amino acids into the formation of essential carbohydrates through gluconeogenesis.

The increased glutamate dehydrogenase (a mitochondrial enzyme) activity in the present study could be due to the acceleration of oxidative deamination of amino acids into the citric acid cycle through α - ketoglutarate or even due to disruption of mitochondrial organization.

Further, at 24, 72, 120, and 168 hours of exposure to high temperature, the observed major metabolic changes in the hemolymph, fat body and silkgland indicated considerable changes in biochemical constituents. In *Bombyx mori*, the fat body is the vital tissue for the synthesis of various biochemical constituents as well as hemolymph and also an active site for the intermediary metabolism of amino acids (Kilby, 1963). Hemolymph which is in direct contact with the fat body and silkgland acts as an intermediate for interchanging various metabolites. Thus, it is reasonable to expect that any changes in metabolic constituents of the hemolymph at high temperature correlates with the disruption of fat body and silkgland.

The major problem in the tropical areas is the extended period of high temperature from late February to July of every year resulting in drastic reduction in cocoon yield. Ueda and Lizuka (1962) demonstrated that silkworms were more sensitive to temperature during the 4th and 5th stages. Overall findings of the present study also revealed that, in general, the effects of direct exposure to high temperature during the 5th instar showed significant reduction in larval duration, larval body weight, cocoon weight, shell weight, fecundity and filament length as compared to the controls. Some of these observations support the findings of the earlier workers (Sugai and Takahashi 1981, Pillai and Krishnaswami 1987). The significant reduction in larval body weight and various economic characters on exposure to high temperature might be due to some physiological disorders within the silkworm body, which is clearly evident from the present studies pertaining to protein metabolism under the influence of high temperature conditions.

## SUMMARY, CONCLUSIONS AND RECOMMENDATIONS

1. In the tissues of $NB_4D_2$ silkworms, total proteins significantly decreased in the hemolymph, fat body, and silkgland of silkworms on exposure of the post IV moult larvae to high temperature. Corresponding to the decrease in total protein levels, there was an increase in protease activity was observed. The results indicated severe proteolysis in the hemolymph, fat body and silkgland of silkworms under high temperature stress conditions. Increase in activity of protease, a lysosomal enzyme, may be due to massive lysis of tissues or even could be due to the damage caused to lysosomes at high temperature. The decrease in proteins could be also

due to the cellular destruction and suppressed protein synthetic potentials.

2. There was decrease in free amino acid pool in the hemolymph, fat body and silkgland which could indicate the mobilization of free amino acids into oxidative metabolism to overcome heat stress conditions.

3. AlAT and AAT activities increased in hemolymph, fat body and silkgland. The increase in these enzyme activities indicate the activation of transdeamination reactions.

4. There was increase in GDH activity in all the tissues studied which could be due to the disruption of mitochondrial organization or may be due to the acceleration of oxidative deamination of amino acids into the citric acid cycle through α-ketoglutarate.

5. The 5th instar larval duration, larval body weight, single cocoon weight, single shell weight, fecundity and average filament length were decreased. This type of reduction may be due to some physiological disorders and due to the lack of protein synthesis pattern wihin the silkworm body under high temperature stress conditions.

6. Since silkworms require constant temperature and relative humidity during their development, we recommend implementation of all possible efforts to reduce the direct temperature impact on silkworms by using wet foam pads during the young age rearing, humidifiers and sand beds with sufficient water during late age rearing to reduce the temperature as well as to the increase relative humidity in the rearing houses. Also more tolerant and more productive breeds/hybrids should be selected and used for the rearing purposes to get high silk yield as well as to avoid crop failures.

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| Milliliter | ml |
| Cubic meter | $m^3$ |
| Energy and Work | KJ |
| Kilojoule (replace calorie in dietetics) | |
| Mass | |
| Kilogram | kg |
| Gram | g |
| Ton (metric ton) | t |
| Milligram | mg |
| Time (same units used in both Metric and English System) | |
| Day | d |
| Hour | h |
| Minute | min |
| Second | s |
| Amount of substance | |
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